DEVELOPMENT OF OPTIMAL METHODS FOR EFFECTIVE DETECTION AND RECOVERY OF INFECTIOUS ENTERIC VIRUSES FROM THE ENVIRONMENTAL WATER

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By
Zi Wang

Thesis Committee:

Yuanan Lu, Chairperson
Alan Katz
Dongmei Li

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ABSTRACT

Human enteric viruses have been used as alternative indicators for water quality monitoring since the current used bacterial indicators do not always corresponded with true health risk. Effective laboratory methods for viral detection and concentration are essential since these human enteric pathogens exist in an extremely low number in environmental water. Our laboratory has recently established a molecular technique, i.e. PCR, for effective concentration and sensitive detection of these viruses. However, this method has limitations for differentiating between infectious and non-infectious viruses. Therefore, this study is aimed to establish optimized laboratory conditions for concentration and recovery of infectious viruses from environmental waters. For effective recovery of concentrated infectious viruses from negatively charged filter membranes, three different elution buffers: NaOH, KH_2PO_4 and beef extract (BE) solution were compared. And for viral isolation and infectivity from shellfish tissues, PEG precipitation method and ultracentrifugation method were compared by using Poliovirus type 1 as a viral model. Viral infectivity was conducted through plaque formation assay and demonstrated that 3% BE in 50 mM glycine is the most effective buffer for virus recovery (>90%). And over 77% of infectious viruses were recovered from shellfish tissue homogenates through PEG 6000 precipitation.

The established viral plaque assay was also used to determine the stability of spiked

Poliovirus 1 under various experimental conditions including seawater, fresh water, PBS, 50% raw sewage, 50% sewage effluent, 100% raw sewage, 100% sewage effluent and also in the indigenous bivalve mollusk tissue. It showed that Poliovirus 1 is less stable in sea water than the other aquatic environments. Viral survival curves revealed no significant difference in the stability of infectious Poliovirus 1 between raw sewage and sewage effluent. However, they illustrated that Poliovirus 1 is more stable in 100% raw sewage/sewage effluent than it in 50% raw sewage/sewage effluent.

Additionally, the indigenous shellfish (*isognomon spp.*) was observed to have an accumulation function for infectious Poliovirus 1 in their tissue and could retain the virus for a longer time than in the surrounding water which enhanced the use of shellfish as a natural bio-indicator for environmental water quality monitoring.

This study has established optimized laboratory conditions for the concentration and recovery of infectious enteric virus and the determination of viral stability in different environments. This provides the essential information for the facilitation of current tests using enteric viruses and shellfish as indicators for aquatic environmental monitoring and for assessing contamination-associated health risks.

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Introduction

1. Background

1.1 Recreational waters contamination and pathogens

Water-borne disease has been reported to relate with recreational water activities since the 1950s (Prüss 1998, Sinclair et al., 2009). There are around 3.5 million people infected by waterborne disease every year due to swimming in contaminated water reported by the United States Environmental Protection Agency (U.S.EPA) (Stoner and Merkel, 2004). As known, there are many different sources resulting in pathogens in recreational water. Besides water runoff and swimmers, the major source is insufficiently treated sewage or sewage overflows discharged by the sewage treatment plants which could contribute to a high risk of contamination in recreational water (U.S.EPA, 2006).

Most of the sewage collection pipes in U.S. are between 50 to 100 years old which convey over 50 trillion gallons of the raw sewage every day (Stoner and Merkel, 2004). Although there are explicit laws regulating the sewage treatment process, these aging pipes may release untreated raw sewage directly into the environment. In addition, population growth and climate changes also put great pressure to the sewage transportation and treatment systems, which may lead to the sewer overflow as a public risk. For example, sewage overflow, or broken sewer pipes was reported as the source of contamination for 74 closing/advisory days — 61% of the year's total at Michigan's beaches in 2002 (Stoner

and Merkel, 2004).

As one of the major pathogens, viruses living in the environmental water were first isolated from water in 1930s. With technique development, more and more pathogenic viruses have been discovered and confirmed to be associated with water contamination. Reports of viral contamination outbreaks in recreational water were increased in the past several years which may be due to the improvement on the detection methods and increasing concerns of Centers for Disease Control and Prevention (CDC). However, a recent article demonstrated that the number of the viral outbreaks actually increased over the past years (Sinclair et al., 2009).

Viruses in the environmental water could directly come from the raw sewage such as sewage overflow, leakage and broken sewer pipes. Besides, the discharge of the incompletely treated sewage would be another important source for the virus contamination. For instance, as a commonly used method to purify wastewater, chlorination would not always work well to eliminate viruses. Moreover, ultraviolet light (UV) was reported to be less effective to disinfect viruses when there are various solids in the sewage water (Stoner and Merkel, 2004). It is well known that human enteric viruses in the environmental water could cause many different diseases, most of which may result in the enteric infections, e.g. gastroenteritis. (Fong and Lipp, 2005; Stone et al., 2004). Table 1 shows the most common enteric viral pathogens in the environmental water and related

diseases (Fong and Lipp, 2005; U.S.EPA, 2006).

Table 1. Diseases and symptoms caused by human enteric viruses.

Pathogens	**Disease**	**Effects**
Adenovirus	Respiratory disease, gastroenteritis and conjunctivitis	Eye infectious, diarrhea
Astrovirus	Gastroenteritis	Diarrhea, fever and vomiting
Calicivirus	Gastroenteritis	Vomiting, diarrhea
Coxsackievirus	Coxsackievirus infections	Fever, Hand, foot and mouth disease and herpangina
Echovirus	Respiratory disease	Sore throat and cough
Hepatitis A and E	Infectious hepatitis	Fever, jaundice and abdominal pain
Norwalk virus	Gastroenteritis	Diarrhea
Poliovirus	Poliomyelitis	Flu-like symptoms and paralysis
Reovirus	Respiratory disease, gastroenteritis	Diarrhea, vomiting
Rotavirus	Gastroenteritis	Diarrhea, vomiting

1.2 Water-borne disease occurrence and epidemiology

Water-borne disease increased constantly in the past decades (Bosch, 1998; Stone et al., 2004; Sinclair et al., 2009). In 1990, Cheung et al. reported that more than 400,000 cases of the water-borne disease resulted from the contaminated beach water in Hong Kong. In a cohort study conducted by Fleisher et al. in 1998, it was shown that an exposure to the marine water contamination for three years might result in 34.5% of gastroenteritis cases and 65.8% of ear infections cases nationwide. In 2002, CDC built a surveillance system to monitor water-borne diseases nationally showing that the sewage contamination led to more than seven million cases of mild infection and more than half million serious infections every year (Reichard et al., 1994). Studies on water-borne disease also illustrated that outbreaks of water-borne gastrointestinal disease related to the recreational water increased consistently from 1989 to 2002, within which the contribution of the viral gastroenteritis went up from 11% to 44% (Sinclair et al., 2009).

The water-borne diseases also have a great impact on economics (Okoh et al., 2010, Stoner and Merkel, 2004). With more and more Americans travelling to the coastal areas, the trend of the recreational activities such as beach visits, swimming, snorkeling, scuba diving and jet skiing, grew from 6% in 1995 to 183% in 2001. The EPA estimates that 28.3 million jobs involving 54 billion dollars are provided by tourism related to the coastal areas every year (Stoner and Merkel, 2004). However, with the increase of recreational water

contaminations, many beaches have to be closed for the etiological investigation. A report showed that over 25,000 beaches were closed due to pollution in 2006 across the country (Christy, 2007) which brought a direct negative impact on the coastal area economy and productivity as well (Stoner and Merkel, 2004; U.S.EPA, 1999). The latest research illustrated that there are several reasons for the increasing outbreaks caused by swimming in the contaminated water as following:

First of all, the population growth brings much pressure on the sewage treatment system, which might lead to an overuse and breakdown of the system. Also, the extreme environmental condition caused by the climate change such as storms, landslides, and typhoons might bring a very high risk of the sewage overflow. It was illustrated that pathogens could be detected from the samples collected from the beach water in Southern California, especially in the rainy seasons (Tsai et al., 1993).

Furthermore, due to the lack of regular recreational water quality monitoring programs at both states and local levels in the U. S., reports of outbreaks might be hidden to the government and the risk of disease might be underestimated (U.S.EPA, 1999). A report of Washington State in 2000 showed that only 6 counties in Washington State had routine monitoring programs for the public beaches while 16 counties rarely monitored the public beach.

In addition, with a better understanding of water-borne disease, epidemiologists have a

clearer idea about the potential experimental bias which might influence the results (Pruss, 1998; Sinclair et al., 2009; U.S.EPA, 1999).

In related epidemiological studies, problems such as length of follow-up, water quality definition, health outcome definition, local condition and tests for internal consistency of results, might need to be overcome. The three major designs of the epidemiological study have been used to investigate water-borne disease outbreaks, whereas the three designs have their own limitations. For instance, loss of follow-up would be a big problem for the prospective studies, while it might be hard to evaluate the water quality at the specific exposure time when conducting the retrospective cohort studies. And the ethical problem is always unavoidable in randomized control trails (Pruss, 1998).

Due to the specificity of the short contacting time and long incubation periods for the water-borne diseases, people may not always correlate their disease to contaminated water (Craun et al., 2005). In some cases, diseases could not be recognized until a secondly person-to-person infection appears (Bosch, 1998). Furthermore, the pathogens in water may result in varying symptoms including different severity levels of infections, which mainly depend on characteristics of the subjects such as age, immune system and route of the infection (Bosch, 1998). Based on the previous studies, it is concluded that outbreaks of water-borne disease were very hard to investigate and monitor due to their diversification from epidemiological thinking.

1.3 Currently used indicators for water quality monitoring

The U.S. EPA has established related criteria to monitor recreational water quality since 1976 (U.S.EPA, 1999) and provided grants to states and local level's pollution control agencies to establish monitoring programs. Since screening for every possible pathogen of concern is unachievable, indicators of human fecal pollution are adopted to monitor water quality. An ideal indicator should be absent in the unpolluted environment, should not multiply when leaving host, should be easily detected by simple laboratory tools and is normally not considered pathogen itself. However, at the present time no indicators in common use meet all these criteria. (Bosch, 1998; Sloat and Ziel, 1992).

In 1976, EPA recommended to use fecal coliforms as the indicator organism. Escherichia coli (E. coli) and enterococci were determined as the indicators in the new guidelines published in 1986 which is still used today. These fecal indicator bacteria (FIB) have been widely used for over 30 years in the U.S. since the established detection method is inexpensive and widely available and FIB could detect potential pathogens under similar environmental conditions (U.S.EPA, 2006). However, there are some limitations associated with FIB which need to be emphasized. Firstly, bacteria indicators fail to distinguish human and animal pathogens which might lead to an overestimate of the illness risk (U.S.EPA 1999). Secondly, bacteria may multiply in water environment without hosts. Another major inadequacy of bacteria indicators is that they are not always consistent with

the viral contamination in waters (Bosch, 1998; Fleisher et al., 1998; Fong and Lipp, 2005; Griffin et al., 2003; Pruss, 1998; Sair et al., 2002; Sinclair et al., 2009). There are many epidemiological studies showing that viral related outbreaks may occur when bacterial indicators are absent, which highlights the necessity of establishing an appropriate monitoring indicator for the viral contamination in environmental waters (Bosch 1998; Okoh et al., 2010; Sinclair et al., 2009).

1.4 Alternative indicators for viral pathogens

As described previously, bacterial indicators are not always reliable for viral contamination detection in the recreational water. Therefore, alternative indicators for viral pathogens in water need to be established. Human enteric viruses including adenoviruses, astroviruses, rotaviruses and enterovirus are the major pathogens in virus associated contaminations in recreational water and are suggested as the potential alternative indicators conjunct with FIB to monitor water quality (Fong and Lipp, 2005; Mcneil et al., 1999). It was reported that enterovirus has already been used as a regular indicator for water monitoring in the European Union countries (Lucena et al., 1986; Puig et al., 1994).Compared to bacteria, human enteric viruses have several specific characteristics as noted below:

The first is that the dose of human enteric viruses for causing disease is much lower than that of the bacteria, in some cases, only one viral unit could have caused infection in human. The dose for 1% chance infection by exposure to 1 poliovirus is 32% to 67%, while only 3%

for rotavirus (Bosch, 1998; Fong and Lipp, 2005). A risk assessment conducted in 1993 showed that the illness risk was much less for bacteria than viruses when the same number of pathogens is exposed (Haas et al., 1993).

Secondly, human enteric viruses are more stable and persistent than bacteria in the environment waters and sewage. It is known that enteric viruses could survival in many extreme situations such as pH value ranging from 3 to 10 and low temperature (Griffin et al., 2003). As described previously, due to their small size, viruses may escape from some wastewater treatment processes such as sedimentation, activated carbon treatment and oxidation ponds (Okoh et al., 2010). It was also shown that the stable DNA or RNA structures of virus could allow them to survive from UV treatment (Gerba et al., 2002). As a conclusion, bacterial indicators degrade faster than viruses in both the environmental water and sewage treatment processes (Bordalo et al., 2002; Solic and Krstulovic , 1992).

Furthermore, unlikely bacteria, human enteric viruses target solely on humans and are not able to multiply in the environment once they leave the host which allow them to be good indicators (Fong and Lipp, 2005).

Last but not least, human enteric viruses may cause serious disease. In addition to gastroenteritis and diarrhea, enteric viruses could cause serious diseases related with other systems of human which are shown in Table 1. In addition, virus related diseases generally have longer incubation period than bacteria-associated disease which make them hard to

be determined (Sair et al., 2002). Although human enteric viruses do not meet all the criteria for an ideal indicator, they have shown a great potentiality to act as indicators for monitoring water quality related to the viral contamination.

1.5 Human enteric virus used in this study

In this study, poliovirus type 1 was used as a viral model for all the experiments. Poliovirus belongs to the family Picornaviridae and the genus of enterovirus, which is a single-stranded RNA virus with 30 nanometres in diameter (John et al., 2005). Poliovirus 1 transmits by fecal-oral route and sometimes may be directly infected by skin contacting. The clinical symptoms of poliovirus infection are not only limited to gastroenteritis, but depend on which organs it infects (Destombes et al., 1997). It is known that Poliovirus firstly attaches and replicates in the gastrointestinal system and once it travels to other organs by blood, it may cause serious diseases such as poliomyelitis (Racaniello, 2006). Although the vaccination of poliovirus has virtually protected people in the U.S., the poliomyelitis is still a serious problem in some developing countries (Cliver, 1997). In the past years, Poliovirus 1 was used as a viral model for virological research due to its wide-existing in human sewage, easily detecting method and available culture cells (Pina et al., 1998; Sair et al., 2002). Therefore, Poliovirus 1 is chosen as viral model in this study.

1.6 Virus detection methods

As a molecular method to detect the target DNA by producing multiple copies, the polymerase chain reaction (PCR) has been used for viral detection since 1980s (Griffin et al., 2003; Toze, 1999). PCR has been widely adopted as the viral detecting method due to its less time consuming and easy operation (Chung et al., 1996; Hafliger et al., 1997). In addition, PCR is able to target to specific DNA if appropriate primers are used, thus, most of the human enteric viruses can be detected by PCR (Griffin et al., 2003; Toze, 1999). Moreover, PCR is highly sensitive in that only 0.1 PFU of virus could be detected (Griffi et al., 2003). Although PCR detection method offers a very useful and fast way for virus detection from environmental waters, it has some disadvantages that may limit its application.

First of all, the polymerase enzyme used in PCR process is sometimes susceptible to other components in water such as human compounds, which might inhibit the PCR process and finally causes a false-negative result (Toze, 1999). Secondly, various contaminations of samples and reagents might cause false-positive results due to the high-sensitivity of PCR (Fong and Lipp, 2005; Toze, 1999).

Last but not least, PCR is not capable to distinguish whether the target virus is infectious or not. It was shown that DNA might keep stable in the cover of a dead virus, even be naked outside the viral particle and directly presents in the water (Toze, 1999). Therefore, there

may have a great possibility that PCR only detects RNA/DNA fragments but not infectious viral particles in the sample (Toze, 1999). This drawback may lead to an overestimate of the result because only infectious virus has pathogenicity and may cause diseases of humans. Therefore, in order to evaluate the true health risk associated with recreational water contamination, this study is aimed to develop an effective concentration and recovery method for infectious virus from environmental waters.

At present, the cell culture is the most recognized laboratorial method to evaluate infectious virus (Griffin et al., 2003). Although studies showed that the results of PCR assay is sometimes consistent with cell culture detection methods (Limsawat and Ohgaki, 1997), it is still proven that PCR may give positive results even if virus become inactive and undetectable by cell culture methods (Leveque et al., 1995). Cell culture was the most widely used method for virus detection before the development of PCR, and is still the best method to evaluate infectious virus from environmental water (Fong and Lipp, 2005). Although the disadvantages of the cell culture are certain, for instance, it needs more labor and consuming time than those of PCR technique (Griffin et al., 2003) and is unable to provide available cell lines for all the virus (Pommepuy and Guyader, 1998), there are no alternative methods to determine infectious viruses in environmental samples at present (Griffin et al., 2003). Based on the above discussion, therefore, cell culture method was employed to detect and evaluate the infectious viruses in this study.

1.7 Shellfish as a natural bio-indicator for water quality monitoring

Bivalve mollusks are animals with two shell valves which are hinged together at one end (Gerba et al., 1978). They are filter-feeding animals that are able to filter up to 1500 L of water every day (Gerba et al., 1978; Winn, 1999). It is shown that when the bivalve mollusks are presenting in polluted water, they can accumulate pathogens via filtering and keep them in the digestive system for a long time. Therefore, shellfish are considered as a vehicle of human enteric viruses (Comelli et al., 2008; Gerba et al., 1978; Lewis et al., 1988).

Some studies demonstrated that the viral occurrence in water could not reflect its true presence in the shellfish growing in the water (Sair et al., 2002; Uhrbrand et al., 2010). It is reported that enteric viruses may present at a much higher level in shellfish than surrounding water (Bosch, 1998), and are still detectable in shellfish tissue for 8 days after disappearing in environmental water (Asahina, 2009). It has been shown that shellfish can concentrate up to 100-fold of virus when comparing with surrounding water (Enriquez and Gerba, 1992) which provide a more sensitive method for infectious viral detection than directly processing the waters. This study is aimed to establish an effective viral extraction and concentration method from shellfish tissue in order to use it as a natural bio-indicator to assess water contamination.

2. Significance

The establishment of rapid and efficient laboratory methods for infectious viral concentration and recovery from environmental water and shellfish was designed to lay a solid base for monitoring infectious virus in recreational water, and support the notion of using human enteric virus as a potential indicator to monitor and assess recreational water quality in the future.

It is noted that although the current recreational water quality criteria which rely solely on the FIB is not always reliable for monitoring infectious viral contamination outbreaks, it is the only monitoring system so far that is based on epidemiological studies for waterborne disease prediction. The finding of our study as an alternative monitoring indicator is considered as complementary and supportive of current system, which provides the initiation step for infectious viral monitoring in recreational waters. The development and utilization of this study which helps to establish an alternative water quality monitoring system for infectious viral detection may provide a better understanding of the prevalence of fecal contamination in the recreational water not only in Hawaii, but worldwide.

3. Specific aims

1. Establishment of optimized laboratory conditions for efficient concentration and elution of infectious Poliovirus 1 from the environmental water.
2. Establishment of optimized laboratory methods for effective extraction and recovery of infectious Poliovirus 1 from tissue of shellfish collected in Hawaii water.
3. Evaluation of stability of Poliovirus 1 in different liquid environmental conditions.
4. Evaluation of the bioaccumulation function of Poliovirus 1 in shellfish tissue under laboratory conditions.

4. References

Bordalo A., Onrassami R. and Dechsakulwatana C. (2002) **Survival of Fecal Indicator Bacteria in Tropical Estuarine Waters (Bangpakong River, Thailand).** *J. Appl. Microbiol.* 93:864-871.

Bosch A. (1998) **Human Enteric Viruses in the Water Environment: a Minireview.** *International Microbiology* (1998)1:191-196.

Cheung, W.H.S., K.C.K. Chang, and R.P.S.Huang. (1990) **Health Effects of Beach Water Pollution in Hong Kong.** *Epidemiol. Infect.* 105:139-162.

Christy Leavitt. (2007) **Troubled Waters: An Analysis of 2005 Clean Water Act Compliance.** *U.S. PIRG. Education Fund.*

Chung H., Kaykus L. and Sobsey M. (1996) **Detection of human Enteric Viruses in Oysters by in Vivo and in Vitro Amplication of Nucleic Acids.** *Appl. Environ. Microbiol.* 62:3772-3778.

Cliver D. O. (1997) **Virus Transmission via Food.** *Food Technol* 51(4): 71-78.

Cordell, H.K. (2008) **The latest on trends in nature-based outdoor recreation**. *Forest History Today. (Spring): 4–10.*

Craun G.F. Calderon R.L. and Craun M.F. (2005) **Outbreaks Associated with Recreational Water in the United States.** *International Journal of Environmental Health Research*. 15(4): 243.

Destombes, J., Couderc, T., Thiesson, D., Girard, S., Wilt, S.G., Blondel, B., (1997) **Persistent Poliovirus Infection in Mouse Motoneurons.** *J. Virol*. 71, 1621 – 1628.

Dziuban E. J., Liang J. L., Craun G. F., Hill V., Yu P. A., Painter J., Moore M. R. Calderon R. L., Roy S. and Beach M. J. (2006) **Surveillance for Waterborne Disease and Outbreaks Associated with Recreational Water --- United States, 2003—2004.** *MMWR Surveil. Summ.* 2006 Dec 22;55(12):1-30.

Fleisher J. M., kay D., Salmon R. L., Jones F. Wyer M. D. and Godfree A. F. (1996) **Marine Waters Contaminated with Domestic Sewage: Nonenteric Illness Associated with bather Exposure in the United Kingdom.** *Am. J. Public health* 86:1228-1234.

Fleisher J.M., Kay D., Wyer M. D. and Godfree A. F. (1998) **Estimates of the Severity of Illnesses Associated with Bathing in Marine Recreational Waters Contaminated with Domestic Sewage.** *International Journal of Epidemiologyl* 1998;27;722-726.

Fong T. T., Griffin D. W. and Lipp E. K. (2005) **Molecular Assays for Targeting Human and Bovine Enteric Viruses in Coastal Waters and Application for Library-independent Source Tracking.** *Appl. Environ. Microbiol.* 71:2070-2078.

Fong T.T. and Lipp E. K. (2005) **Enteric Viruses of Human and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools.** *Microbiology and molecular biology review*, June 2005, p. 357-371.

Gerba C.P., Gramos D.M. and nwachuku N. (2002) **Comparative inactivation of enteroviruses and adenovirus 2 by UV light.** *Appl. Environ. Microbiol.* 68:5167-5169.

Griffin D.W., Donaldson K. A., Paul J. H. and Rose J. B. (2003) **Pathogenic Human Viruses in Coastal Waters.** *Clinical Microbiology Reviews*, Jan. 2003., p. 129-143.

Haas C.N., Rose J.B., Gerba C.P. and Stig R. (1993) **Risk Assessment of Viruses in Drinking Water.** *Risk Analysis* 13:545-552.

Hafliger D., Gligen M., Luthy J., and Hubner P. (1997) **Seminested RT-PCR Systems for Small Round Structured Viruses and Enteric Viruses Detection in Seafood.** *Int. J. Food Microbiol.* 37:27-36.

Henrickson S. E., Wong T., Allen P., Ford T. and Epstein P. R. (2001) **Marine Swimming-related Illness: Implications for Monitoring and Environmental Policy.** *Environ. Health Perspect.* 109:645-650.

John K. Daley, Lisa A. Gechman, Jason Skipworth and Glenn F. Rall. (2005) **Poliovirus Replication and Spread in Primary Neuron Cultures.** *Virology* 340 (2005) 10 – 20.

Leveque F., Crance J. K., Beril C. and Schwartzbrod L. (1995) **Virucidal Effect of UV Light on Hepatitis A Virus in Seawater-Evaluation with Cell Culture and PT-PCR.** *Water Sci. Technol.* 31,157-160.

Limsawat S., and Ohgaki S. (1997) **Fate of Liberated Viral RNA in Wastewater Determined by PCR.** *Appl. Environ. Microbiol.* 63:2932-2933.

Lucena F., Schwartzbrod L. and Bosch A. (1986) **The Effect of a Mass Poliomyelitis Vaccination Program on the Occurrence of Enterovirus in Sea Water.** *Zentbl. Bakteriol. Hyg.B.* 183:67-69.

Mcneil K. M., Hendrix R. M., Linder J. L., Benton R. R., Monteith S. C., Tuchscherer M. A., Gray G. C. and Gaydos J.C. (1999) **Large, Persistent Epidemic of Adenovirus Type 4- Associated Acute Respiratory Disease in U.S. Army Trainess.** *Emerg. Infect. Dis.* 5:798-801.

Mezzanotte V., Antinelli M., Citterio S. and Nurizzo C. (2007) **Wastewater disinfection alternatives: Chlorine, Ozone, Peracetic Acid, and UV Light.** *Water Environ. Res.* 79,2372-2379.

Nobles R. E., Brown J., Rose J. and Lipp K. (2000) **The Investigation and Analysis of Swimming-associated Illness Using the Fecal Indicator *Enterococcus* in Southern Florida's Marine Water.** *Fla. J. Environ. Health* 169: 13-19.

Okoh A. I., Sibanda T. and Gusha S. S. (2010) **Inadequately Treated Wastewater as a Source of Human Enteric Viruses in the Environment.** *International journal of environmental research.* 2010,7,2620-2637.

Pina S., Puig M., Lucena F., Jofre J. and Girones R. (1998) **Viral Pollution in the Environment and in Shellfish: Human Adenovirus Detection by PCR as an Index of human Viruses.** *Appl. Environ. Microbiol.* 64:3376-3382.

Pommepuy M. and Guyader F. L. (1998) **Molecular Approaches to Measuring Microbial Marine Pollution.** *Curr. Opin. Biotechnol.* 9:292-299.

Pruss A. (1998) **Review of Epidemiological Studies on Health Effects from Exposure to Recreational Water.** *International Journal of Epidemiology* 1998:27 1-9.

Puig M., Jofre J., Lucena F., Allard A., Wadell G. and Girones R. (1994) **Detection of Adenoviruses and Enteroviruses in Polluted Waters by Nested-PCR Amplification.** *Appl. Environ. Microbiol.* 60:2963-2970.

Racaniello V.R. (2006) **One hundred Years of Poliovirus Pathogenesis.** *Virology* 344 9-16.

Reichard E., Zapponi G. (1994) **Assessing and Managing Health Risks from Drinking Water Contamination.** *Proceeding's of International Symposium in Rome, Italy, Sep. 1*3-17.

Review. *Journal of applied microbiology* 107(2009) 1769-1780.

Sair A.I., Souza D.H. and Jaykus L.A. (2002) **Human Enteric Viruses as Causes of Food-borne Disease.** *Comprehensive reviews in food science and food safety. 1(2) 73-89.*

Sinclair R.G., Jones E.L. and Gerba C.P. (2009) **Viruses in Recreational Water-borne Disease Outbreaks: a Review.** *Journal of applied microbiology* (2009) Dec 1;107(6):1769-80.

Sloat, S., and C. Ziel. (1992) **The Use of Indicator Organisms to Assess Public Water Safety.** *Hach Company, Loveland, CO.*

Solic M. and Krstulovic N. (1992) **Separate and Combined Effects of Solar Radiation, Temperature, Salinity, and pH on the Survival of Fecal Coliforms in Sea Water.** *Mar. Poll. Bull.* 24:411-416.

Stoner N. and Merkel M. (2004) **Swimming in Sewage.** *Natural Resources Defense Council.*

Toze. S. (1999) **PCR and the Detection of Microbial Pathogens in Water and Wastewater.** *Water resourse.* Vol. 33, Mo.17,pp 3545-3556.

Tsai Y. L., Sobsey M. D., Sangermano L.R. and Palmer C.J. (1993) **Simple method of concentrating enteroviruses and hepatitis A virus from sewage and ocean water for rapid detection by reverse transcriptase-polymerase chain reaction.** *Appl.Environ. Microbiol.* 59:3488-3491.

U.S. EPA (Environmental Protection Agency) (1983) **Health effects criteria for marine recreational water.**

U.S. EPA (Environmental Protection Agency) (1984) **Health effects criteria for fresh recreational water.**

U.S.EPA (Environmental Protection Agency) (1999) **EPA Action Plan for Beaches and Recreational Waters.**

U.S.EPA (Environmental Protection Agency) (2006) **Health Concerns at Beaches.** Electronic resources. Retrieved from http://water.epa.gov/type/oceb/beaches/upload/2007_02_13_beaches_report_full-rtc.pdf

Chapter 2

Establishment of optimized laboratory conditions for efficient concentration and elution of infectious poliovirus 1 from environmental waters

1. Introduction

Human enteric viruses have been proposed as alternative indicators for water quality monitoring. However, due to their extremely low number presenting in the environmental water, the development of an effective concentration and sensitive detection method is urgently needed.

There are a variety of methods for viral concentration from water which can be generally classified into three major categories by rationale: Adsorption-elution method, precipitation method and ultracentrifugation method (Bosh, 1998). Among these methods, adsorption-elution is widely applied to detect the viral contamination and was adopted as a standard assay for the drinking water monitoring by U.S. EPA in 1996 due to its high recovery rate and convenient process.

The adsorption-elution method is designed as a filtration system with filter membranes and elution buffers which are applied to adsorb and elute the viral particles, respectively. Katayama et al. in 2002 developed a viral concentration and elution method which is passing spiked sea water through a type-HA negatively charged membrane and eluting the viruses with 1 mM NaOH. Based on his study, the cations such as Mg^{2+} and Al^{3+} in the

seawater would bind the virus to the negatively charged membranes due to electrostatic interaction. After an acid rinse step, the viral net charge would convert to positive which permit the virus to attach to the membrane directly. Once the alkaline elution buffer is applied, the virus would convert to net negative charged and be eluted out from the membranes (Patricia et al., 1983).

This method has been shown to be a very effective way to concentrate viruses from large volume of water by PCR. However, as described previously, PCR could not differentiate between infectious and non-infectious viruses, and few studies have been done focusing on concentrating infectious viruses from the water (Haramoto et al., 2009). Therefore, this study is designed to establish optimal laboratory conditions by evaluating different elution buffers and protocols currently in use for infectious viral concentration and elution to maximize recovery of infectious viruses from environmental water samples.

2. Materials and methods

2.1 Comparison of different water sample elution processes.

2.1a Poliovirus 1 spiked, type-HA membrane filtration and acid rinse

To establish an efficient method to concentrate infectious viruses from sea water, 1 ml poliovirus 1 stock with a viral titer of 1.1×10^6 FPU/ml was spiked into 1 liter pure seawater (from Waikiki aquarium, Honolulu) to obtain a concentration of 1.1×10^3 FPU/ml. Sea water was then concentrated by a filtration system modified based on Katayama et al.'s

study in 2002, which was composed with 2 L-volume filtration funnels, the manifold, the negative charged type-HA filter membranes (Millipore Corporation, MA) with 0.45 um pore size and 47 mm in diameter and the vacuum pump. The seawater was pumped through the type-HA membrane after adding $MgCl_2$ with a final concentration of 25 mM. The membranes were then rinsed with 200 ml 0.5 mM H_2SO_4 (pH 3.0) to remove cations.

2.1b Filtration-elution from membrane

The membranes were removed together with filtration units and transferred upon same sized sterile flasks. 5 ml of the elution buffer was added into the filtration funnel and contacted with the membranes for 5 minutes. After filtering through the membranes by pumping, the eluant was collected into the flasks and then transferred to 15 ml centrifugation tubes. Three selected elution buffers: 1 mM NaOH (pH 10.8), 3% Beef Extract in 0.05 M glycine (pH 9.5) and 0.05 M KH_2PO_4, 1 M NaCl (pH 9.2) were compared to define the optimal effective procedure for recovery of infectious virus. For elution buffer NaOH, 1 ml 10X TE buffer (pH 8.0) and 50 ul 100 mM H_2SO_4 (pH 1.0) were prior added into the flasks to neutralize NaOH.

2.1c Elution through membrane shaking

After the acid rinse, the membranes were removed from the filtration system, and cut into three equal pieces with sterile scissors, then transferred to three 15 ml centrifuge tubes containing three different elution buffers respectively. The 15 ml centrifuge tubes were

then shook on a vortex adapter combined with an analog vortex mixer (VWR, CAT No. 588160121, U.S.A) at speed = 6 for 30 minutes at 4°C. The eluant was then centrifuged at 3000 rpm for 5 min and supernatant was collected and stored at -80°C until use.

2.2 Comparison of different elution buffers and concentrations

Three membranes were processed at the same time in order to compare different concentrations of three elution buffers. After the acid rinse, the membranes were cut into nine identical pieces and applied with different concentrations of the elution buffers: 1 mM, 5 mM, 7 mM of NaOH, 1%, 3%, 5% of Beef Extract, and 1 mM, 5 mM, 10 mM KH_2PO_4 , respectively.

2.3 Optimization of method through membrane shaking

The optimal condition was also determined by comparing different shaking time periods (10min, 20min, 30min, 60min, 90min and 120min). After filtering through spiked sea water and the acid rinse, the membrane was transferred into a 15 ml centrifugation tube containing 5 ml of the optimal elution buffer and shook on a vortex mixer at speed 6. 0.5 ml of the eluant was collected from the centrifugation tube at each selected time point: 10 min, 20 min, 30 min, 60 min, 90 min and 120 min. The eluant was then centrifuged at 3000 rpm for 5 min and supernatant was collected and stored at -80°C until use.

2.4 Cell culture-based infectivity assay-plaque assay

2.4a Cell culture and passage

All operations were conducted inside a cell-culture hood (SterilGARD III Advance°, The Baker Company, Sandford, ME). Green Africa monkey kidney Vero cells (ATCC, Manassas, VA, Cat, No. CCL-81TM) were used for all experiments. Cells were cultured with MEM medium (Minimum Essential Medium Eagle, Manassas, VA 20109), 10% heat-inactivated FBS (fetal bovine serum, HyClone, UT) and 1% PS solution (100U/ml penicillin, 100U/ml streptomycin sulfate) in T-75 cm^2 cultural flasks (Greiner Bio-One, Germany) in a humidified 5.0% CO_2 incubator at 37°C.

Vero cells were passed every 3-4 days by trypsinizing with 5 ml of trypsin-versine solution [10 ml 10X Trypsin (Sigma-Aldrich, St. Louis, MO), 0.2 g EDTP (Sigma-Aldrich, MO), 0.01 g phenol red (Sigma-Aldrich, MO), 0.2 g KCL (Sigma-Aldrich, MO), 1.15 g Na_2HPO_4 (Sigma-Aldrich, MO), and 1 L ddH_2O], after incubating for 3 minutes, the cell monolayer was detached from the flask, the sides of the flask were tapped to remove any remaining adherent cells. Then cell suspension was removed from the flask to a 15 ml centrifuge tube followed by centrifuging at 1,000 rpm for 5 minutes. The supernatant was discarded and pellet was resuspended in 3.5 ml MEM medium. 0.5 ml of the cell suspensions was placed back into the flask again and MEM medium was added to a final volume of 10 ml. The flask was placed back into the incubator at 37°C with 5.0% CO_2. The pH value of medium in flasks was adjusted by HEPES buffer (Mediatech, VA) or 7.5% (w/v) $NaHCO_3$ (Mediatech, VA).

2.4b Viral isolates

Poliovirus 1 was originally obtained from Dr. Philip C. Loh (University of Hawaii, Manoa). Poliovirus 1 was propagated in Vero cells in this study. All the experimental tests involving the use of viruses were performed in a cell culture hood (Labconco Purifier Class II Biosafety Cabinet Delta Series, Labconco, MO) equipped with a UV sterilization lamp.

2.4c Viruses propagation

Vero cells were passed as previously described, after cell monolayer has formed, medium was removed from the flasks within 24 hours. 1 ml of 10^3 to 10^4 FPU/ml Poliovirus stock was immediately added into the flask. The flask was then incubated at 37°C for 1 hour with gently rocking side-to-side every fifteen minutes. The inoculum was then removed, instead, 10 ml of MEM medium with 2% FBS was added into the flask. Then the flask was incubated for 24 hours at 37°C to allow poliovirus infecting Vero cells. When 90% of the cell appeared cytopathic effects (CPE), the flask was immediately transferred into -80°C refrigerator and stored until the medium was frozen. The flask was then thawed at room temperature. After the medium was completely thawed, the flask was placed into -80°C refrigerator again. The freezing-thawing process was repeated three times to release viruses from the cells. The content was transferred to a 15 ml centrifuge tube and centrifuged at 3,000 rpm for 5 minutes. The supernatant was finally collected and aliquoted at 0.5 ml/tube in 1.5 ml centrifugation tubes. All the viral aliquots were stored in -80°C

refrigerator, and the titer of viral aliquots was determined by plaque assay.

2.4d Samples pretreatment

All samples were pretreated with Antibiotic Incubation Medium (2 mM P/S/G, 25ug/ml Amp B, and 500ug/ml Gentamicin, with 1x MEM medium) (AIM) at ratio of 1:2 and incubated at 4°C for 2-4 hours before applying plaque assay.

2.4e Cell plating

The cells were passed as previously described, and the cell suspension was counted on a hemacytometer (Hausser Scientific, PA) then seeded into a 6-well plate at 3×10^5 cells/well. The plate was then incubated at 37°C for 24 hours until a 90% cell monolayer was formatted.

2.4f Viral serial dilutions preparation

Before infection, 10-fold dilutions of Poliovirus were prepared in FBS-free MEM from 10^{-1} to 10^{-8}. Basically, 100 ul of viral sample was mixed with 900 ul of FBS-free MEM. Each serial dilution was made by mixing 100 ul of the previous dilution with 900 ul of FBS-free MEM.

2.4g Virus infection

The medium in 6-well plate was aspirated, and serial viral dilutions were added into wells at 200 ul/well. (Figure 1). One of the wells in each plate was considered as the negative control by adding 100 ul of FBS-free MEM only. The plate was then incubated at 37°C for

1-2 hours for viral adsorption to the cells. Within this time period, the plate was gently rocked from side to side every fifteen minutes. After virus absorption period, the viral dilutions were removed from the wells, 2 ml of the Agar (Sparks, MD21151, U.S.A. Cat. No. 0140-01) and MEM with 2% FBS mixture were added to each well. The plate was incubated for 24 hours to allow cytopathic effect (CPE) to appear. Until CPE was observed, crystal violet staining solution [6.0g crystal violet (Sigma-Aldrich, St. Louis, Mo, Cat. No. C-3886), 400 ml formaldehyde 37% w/w (Sigma-Aldrich, St. Louis, Mo, Cat. No. 252549-4L) and 600 ml ddH_2O)] was added to each well and the plate was stained at room temperature for 24 hours.

2.4h Viral recovery yield calculation

Plaques were counted visually and used to calculate the virus titer. Virus Titer (PFU/ml) = [# plaques counted X dilution factor] / amount of virus dilution used.

2.4i Samples process

All the samples were evaluated by plaque assays to determine the viral titer. 12-well plates were used for water samples infectivity assay and for each dilution, 3 wells were repeated.

2.5 Data analysis

By using SPSS software, one-way ANOVA was performed to all the data of plaque assays, with a p-value equals 0.05.

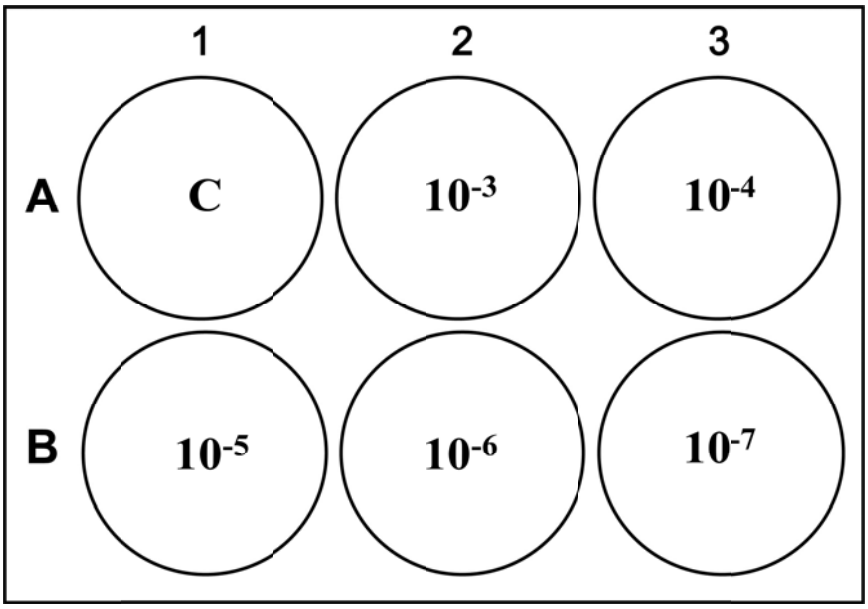

Figure 1. Plating scheme for plaque assay.

3. Results

3.1 Evaluation of Poliovirus 1 stock titer

In this study, the Poliovirus type 1 isolated from the human species was used as viral stock. The titer of the Poliovirus stock was experimentally determined by plaque assay. Figure 2 shows the poliovirus-induced Vero cells in the 6-well plate indicating a linear correlation between the virus dilution and the viral plaque production.

3.2 Vero cells infection by Poliovirus 1

Figure 3 displays the result of the infected Vero cells after 24 hours incubation in a humidified 5.0% CO_2 incubator at 37°C. Obviously, Vero cells have appeared CPE after 24 hours incubation which indicates that Vero cells are susceptible by Poliovirus Type 1.

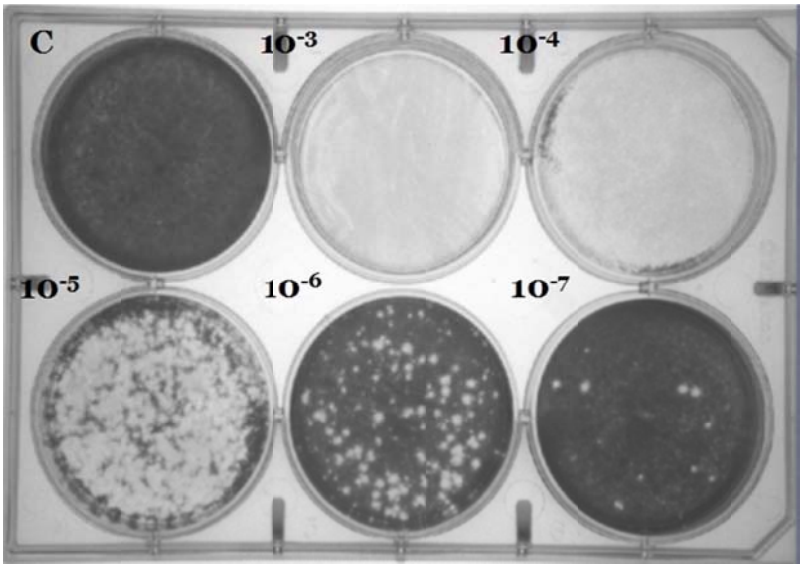

Figure 2. Poliovirus stock titer performed in 6-well plate of Vero cells.

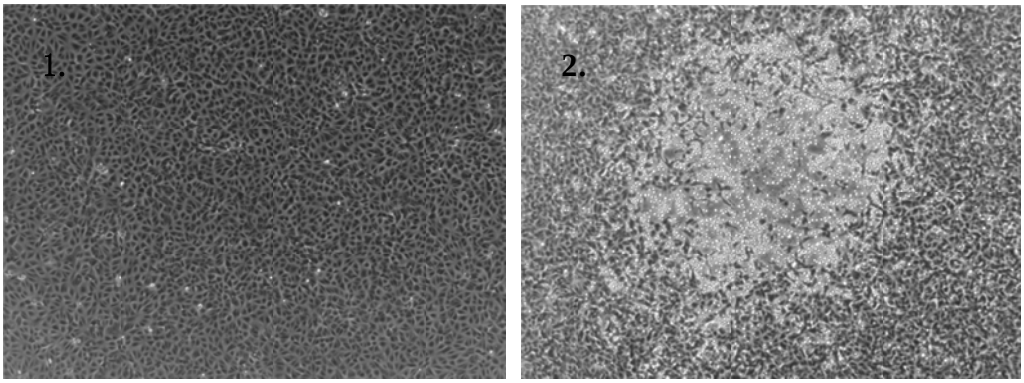

Figure 3. Vero cells pre- and post-infection with Poliovirus 1. **1**: Vero cells before infection **2**: Vero cells 24 hours after infection

3.3 Comparison of two viral elution methods and three elution buffers

Table 2 and Figure 4 illustrate the comparison of the recovery rate under the filtration-elution and shaking elution methods with three different elution buffers. It is found that all the three elution buffers have a similar recovery rate around 26% where $p = 0.921$ indicating no significant difference among three elution buffers by using the filtration-elution method. In comparison, the recovery rate prominently increases to 91.07±8.92% by applying the shaking method with beef extract. Table 2 also shows that no more than 28% of infectious viruses are recovered by NaOH, which indicates that NaOH may not perform a good function for the viral elution for both filtration-elution and shaking method. There is no CPE observed in Figure 4 when KH_2PO_4 was applied in the shaking elution method which demonstrates that KH_2PO_4 might not be effective for the infectious viral elution under such condition.

3.4 Laboratory conditions optimization for shaking elution method

3.4a Comparison of different concentrations of elution buffers

Table 3 and Figure 5 show the comparison of different concentrations of each elution buffer under shaking elution condition. It is found that 1 mM NaOH could yield a higher recovery rate (15.69±0.92%) than that of other concentrations of NaOH ($p < 0.001$). However, the recovery rates for all the concentrations of NaOH are below 20% which indicates that only less than 20% of infectious Poliovirus 1 is recovered from the sea water

by NaOH. At the meantime, Figure 5 illustrates that the recovery rate of 3% Beef Extract is 91.08±8.92%, which is much better than that of 1% Beef Extract ($p = 0.001$), but not significant difference with 5% Beef Extract ($p = 0.721$). Combining the results under elution buffers of NaOH and KH_2PO_4 at different concentrations, it reaches a conclusion that 3% Beef Extract is considered as the most effective elution buffer for eluting Poliovirus 1 from the sea water.

3.4b Comparison of different shaking time periods

Table 4 and Figure 6 display the recovery rate of different shaking time periods by using the shaking elution method. Although there is no significant difference among the recovery rates of shaking time period from 20 minutes to 90 minutes ($p = 0.543$), an overall p-value of 0.048 indicated that they are significantly higher than the other time periods. It is found that the recovery rate is 90±8.4% for 30 minutes period which is the highest among all the compared periods indicating that 30 minutes would be the most effective shaking time period.

Table 2. Comparison of different elution methods and elution buffers for Poliovirus 1 from sea water.

	Filtration-Elution Method			Shaking Elution Method		
Elution Buffer	**Mean Titer (PFU/ml)**	**Virus Input (PFU/ml)**	**Recovery Rate (%)**	**Mean Titer (PFU/ml)**	**Virus Input (PFU/ml)**	**Recovery Rate (%)**
1mM NaOH	1.62×10^6	5.96×10^6	27.13±8.04	2.40×10^4	1.53×10^5	15.69±0.92
3% BE	1.87×10^6	7.15×10^6	26.11±4.98	1.63×10^5	1.83×10^5	91.07±8.92
50mM KH_2PO_4	1.85×10^6	7.15×10^6	25.87±2.62	0	1.83×10^5	0

Table 3. Comparison of different concentrations of three elution buffers.

Elution Buffer	**Concentration**	**Mean Titer (PFU/ml)**	**Recovery Rate (%)**
NaOH	1 mM	2.40×10^4	15.69±0.92
	5 mM	1.87×10^4	12.20±2.01
	7 mM	0.62×10^4	4.03±0.96
Beef Extract	1%	1.17×10^5	63.75±8.92
	3%	1.63×10^5	91.08±8.92
	5%	1.58×10^5	86.52±12.18
KH_2PO_4	10 mM	0	0
	50 mM	0	0
	100 mM	0	0

Table 4. Optimization of membrane shaking time in 3% Beef Extract.

Shaking time	Mean Titer (FPU/ml)	Recovery (%)
Stock	4.67×10^5	/
10 min	3.07×10^5	0.67±0.025
20 min	4.07×10^5	0.87±0.065
30 min	4.20×10^5	0.90±0.084
60 min	3.97×10^5	0.85±0.059
90 min	3.47×10^5	0.74±0.061
120 min	2.87×10^5	0.61±0.038

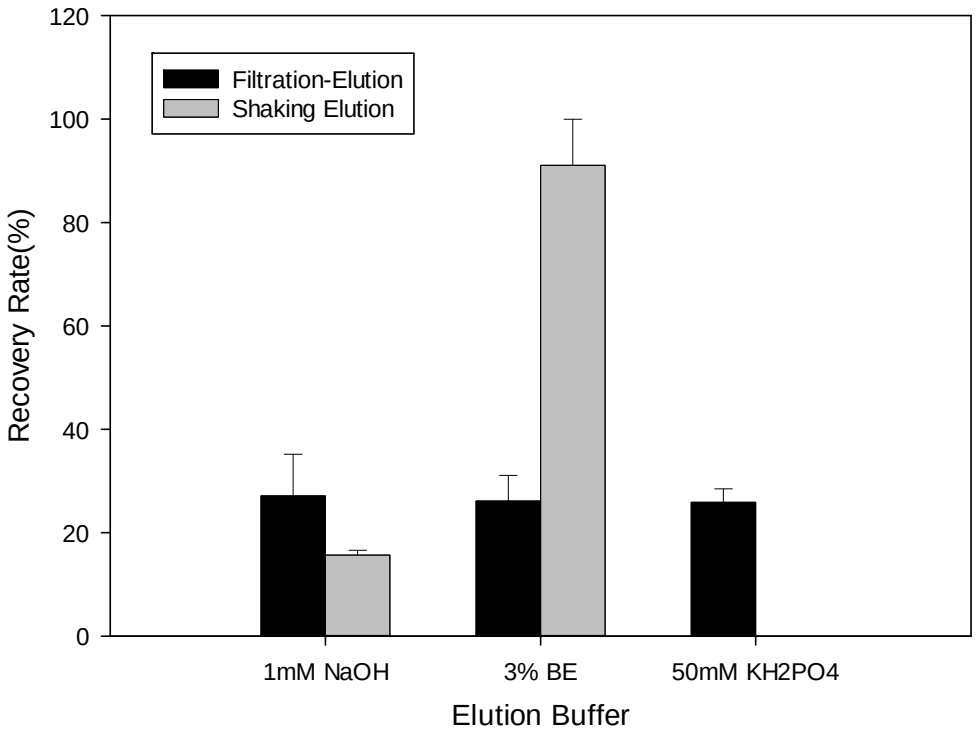

Figure 4. Graph of different elution methods and elution buffers comparison for Poliovirus 1 from sea water.

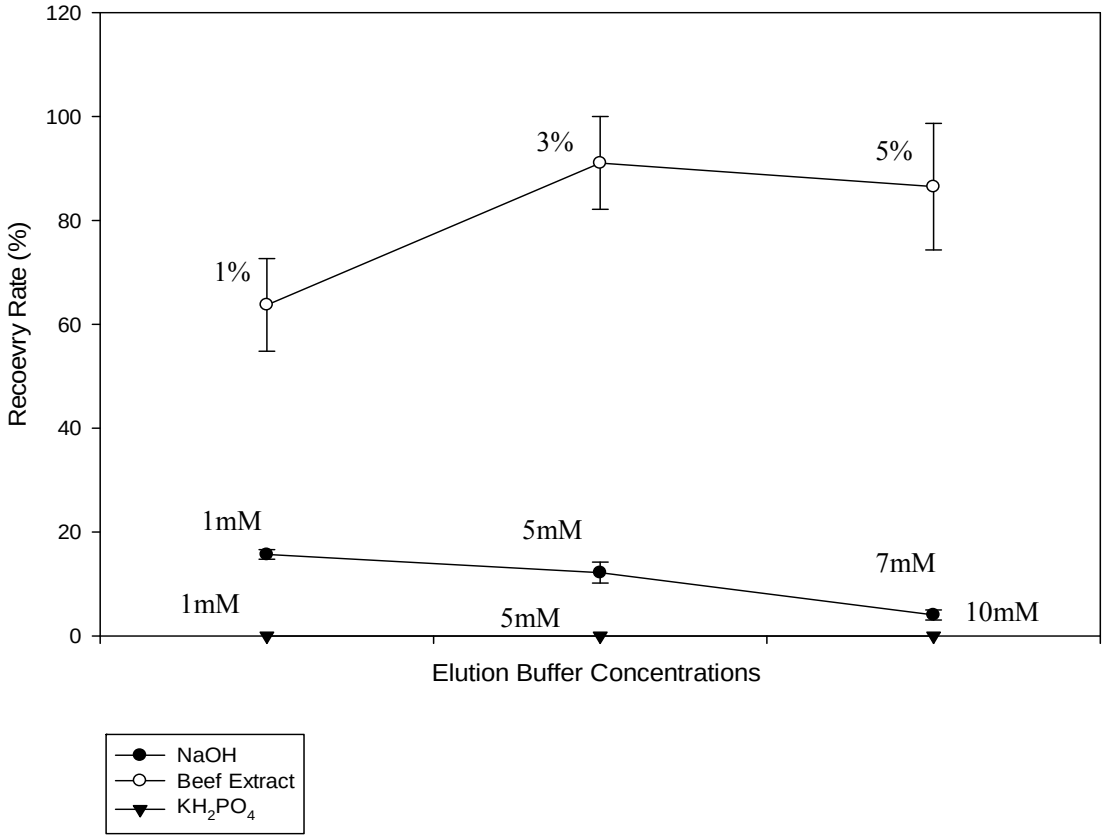

Figure 5. Comparison of different concentrations of elution buffers.

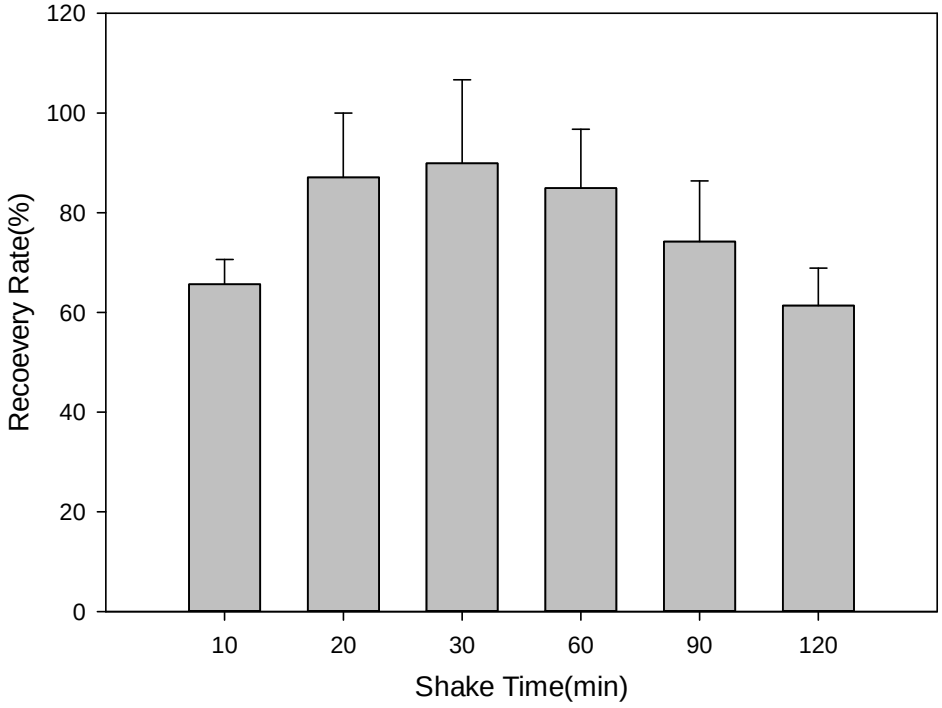

Figure 6. Graph of optimization of membrane shaking time in 3% Beef Extract.

4. Discussion

Current studies on detection of the viruses from water are usually largely dependent on PCR techniques to detect the viral nucleic acid. However, PCR method could not be used for the detection of infectious viruses since it cannot distinguish between the infectious and non-infectious virus as mentioned in the introduction. Since even one infectious viral particle may be able to cause human disease (Bosch, 1998), it is an urgent need for establishing a sensitive method for effective concentration of infectious viruses from water. In order to concentrate the infectious virus from water, we improved the method developed by Katayama et al., in 2002 and the newly developed laboratory protocol was able to recover more than 90% of the infectious Poliovirus Type 1 from the sea water indicating the effectiveness of this optimized method for infectious viral concentration from water.

As shown in Table 2, the filtration-elution method is able to elute about 26% of the infectious viruses from the sea water while the shaking elution method obtains a value as high as 91%. Per discussion on the methodology, it seems that the increase in the recovery rate between these two methods results from the increase of the contacting time between small volume of elution buffers and membranes. In fact, it only took less than 1 minute for 5 ml of the elution buffer to go through the membrane in the filtration-elution method while the shaking elution allowed the elution buffer to contact the membrane for at least 30 minutes.

The shaking elution method used in this study could elute and recover 91% of infectious Poliovirus 1 from sea water. However, in Katayama et al.'s study, it showed a 94% recovery rate for the same case but using QPCR method. As described previously, QPCR can provide a more sensitive quantitative result but it cannot distinguish infectious virus from non-infectious virus, while the cell culture technique we used could directly address this problem by showing the recovery of infectious virus. Based on the present findings from our experimental tests, this study demonstrated that the shaking elution method developed in this study could be applied to effectively elute and recover the infectious virus from the sea water samples.

Among the selected elution buffers, 3% beef extract in 0.05M glycine was determined to be the most effective elution buffer in comparison with those of NaOH and KH_2PO_4, which is consistent with the previous reports (Brassard et al., 2005; Monpoeho et al., 2001). It is also found that 30 minutes was determined as the most effective time period for shaking membrane in 5 ml 3% of Beef Extract. Our result indicates that the shaking time period less than 30 minutes might not be sufficient enough to elute out all the virus particles from the membrane, while the period greater than 30 minutes would not allow to get high recovery efficiency which may be due to the partial damage of the membrane and viral aggregation to membrane debris.

Our study also showed that KH_2PO_4 is a poor elution buffer with a very low efficiency in

virus recovery. It was reported that some solutions fail to elute viruses from membrane at high pH value which is difficult to explain simply by electrostatic interactions between virus and membranes (Farrah et al., 1981). The hydrophobic interaction also plays an important role in the process of viral elution from membranes. Shields et al. described in 1982 that KH_2PO_4 is a kind of antichaotropic ion, which could protect hydrophobic interaction, thereby electrostatic interaction between virus and membranes could not be disrupted and the elution of infectious virus from the membrane is minimal.

5. References

Brassard J., Seyer K., Houde A., Simard C. and Trottier Y. (2005) **Concentration and detection of Hepatitis A Virus and Rotavirus in Spring water Samples by Revese transcription-PCR.** *J. Virological Method* 123:163-169.

Enriquez C. E. and Gerba C. P. (1995) **Concentration of Enteric Adenovirus 40 from Tap, Sea and Waste Water.** *Water Res.* 29:2554-2560.

Farrah S. R. Shah D. O. and Ingram L. O. (1981) **Effects of chaotropic and antuchaotropic agents on elution of poliovirus adsorbed on membrane filters.** *Proceedings of the National Academy of Science.* Vol. 78, No. 2, pp. 1229-1232.

Hamza I. A., Jurzik L., Stang A., Sure K., Uberla K. and Wilhelm M. (2009) **Detection of Human Viruses in Rivers of a Densly-populated Area in Germany Using a Virus Adsorption Elution Method Optimized for PCR Analyses.** *Wat. Res.* 42:2657-2668.

Hamza I. A., Jurzik L., Wilhelm M. and Uberla K. (2009) **Detection and quantification of Human Bocavirus in River Water.** *Journal of General Virology* 90:2634-2637.

Haramoto E., Katayama H. and Ohgaki S. (2004) **Detection of Noroviruses in Tap water in Japan by Means of a New Method for Concentrating Enteric Viruses in Large Volumes of Freshwater.** *App. Environ. Microbiol.* 2004:2154-2160.

Haramoto E., Katayama H., Utagawa E. and Ohgaki S. (2009) **Recovery of Human Norovirus from Water by Virus Concentration Methods.** *Journal of Virological Methods* 160:206-209.

Katayama H., Shimasaki A. and Ohgaki S. (2002) **Development of a Virus Concentration Method and Its Application to Detection of Enterovirus and Norwalk Virus from Coastal Seawater.** *App. Ebviron. Microbiol.* 2002:1033-1039.

Lipp E. K., Lukasik J. and Rose J. B. (2001) **Human Enteric Viruses and Parasites in the Marine Environment.** *Methods Microbiol.* 30:559-588.

Loh P. C. and Fujioka R. S. (1980) **Viruses in Water: Their Detection, Survival and Disease Potential.** *In Environmental Survey Techniques for Coastal Water Assessment Conference Proceedings,* PP. 41-69. UNIHI-SEAGRANT-CR-80-01. Also University of Hawaii Sea Grant College Program, Honolulu.

Lukasik J., Scott T. M., Andryshak D. and Farrah S. R. (2000) **Influence of Salts on Virus Adsorption to Microporous Filters.** *Appl. Environ. Microbiol.* 66:2914-2920.

Monpoeho S., Maul A., Cadiergues B., Schwartzbrod L., Billaudel S. and Ferre V. (2001)

Best Viral Elution Method Available for Quatification of Enterovirus in Sludge by Both Cell Culture and Reverse Transcription-PCR. *App. Environ. Microbiol.* 2001:2484-2488.

Rigotto C., Kolesmikovas C. K., Moresco V., Simoes C. M. O. and Barardi C. R. M. (2009) **Evaluation of HA Negatively Charged Membranes in the Recovery of Human Adenovirus and Hepatitis A Virus in Different Water Matrices.** *Rio De janeiro,* 104(7): 970-974.

Shields P. A. and Farrah S. R. (1982) I**nfluence of Salts on Electrostatic Interactions Between Poliovirus and Membrane Filters.** *Applied and Environmental Microbiology*, Feb. 1983, p. 526-531.

U.S. EPA (Environmental Protection Agency) (1996) **ICR Microbial Laboratory Manual.** EPA 600R95178.

U.S.EPA (Environmental Protection Agency) (2001) **Manual of Methods for Virology.** *EPA/600/4-84/013.* Retrieved from http://www.epa.gov/nerlcwww/about.html.

Chapter 3

Establishment of optimized laboratory methods for effective extraction and recovery of infectious poliovirus 1 from tissue of shellfish collected in Hawaiian waters

1. Introduction

From 1960s, many methods have been developed for extracting and concentrating infectious viruses from shellfish tissue such as acid adsorption, alkaline buffers elution, proteinase K method, polyethylene glycol (PEG) precipitation and ultracentrifugation (Comelli et al., 2008; Lewis et al., 1988; Loisy et al., 2005; Sobsey et al., 1975).

However, most of the methods focus on extraction of viral nucleic acid and involve some process which may destroy and inactivate viable virus, making them inappropriate for concentrating infectious virus. At present, PEG precipitation and ultracentrifugation have proven as the most effective methods used for infectious viral concentration from shellfish tissue (Lewis et al., 1988; Myrmel et al., 2004; Uhrbrand et al., 2010). Therefore, these two methods have been chosen in this study for concentrating Poliovirus 1 in the indigenous shellfish (*isognomon spp.*)

PEGs are water-soluble, non-ionic polymers that are able to attract viral particles and help to separate them from shellfish tissue (Vajda, 1978), while the ultracentrifugation method is aimed to directly centrifuge virus down by using extremely high speeds. Both of the two

methods have been evaluated and optimized in this study to yield a high recovery rate.

2. Materials and Methods

2.1 Shellfish sampling and process

Bivalves *isognomon spp.* Were collected from Kahala Beach, Honolulu. The outer shells were wiped thoroughly with 70% isopropyl alcohol and then shucked by a sterile knife. Shellfish tissue was weighted and collected in a 50 ml centrifuge tube at 5 grams/tube.

2.2 Different virus concentration methods comparison

2.2a Ultracentrifugation

Five grams tissue was blended for 30 seconds with the Brinkman blender (Polytron PT-MR 3000, Littau-Switzerl) after diluted at ratio 1:7 (w/v) with 10% tryptose phosphate broth/0.05M glycine buffer (pH9.5). The homogenate was spiked with 1 ml of 1 x 10^5 to 10^6 FPU/ml of Poliovirus 1, then transferred into a 50 ml beaker and stirred on ice for 1 hour followed by centrifuging at 10,000 x g for 20 minutes at 4°C. The supernatant was collected and adjusted the pH value to 7.0-7.5, followed by ultracentrifuging at 44,000 rpm for 2 hours at 4°C by using a Beckman 70.1 TI rotor. The supernatant of the ultracentrifugation was discarded and the pellet was resuspended in 5 ml PBS. The final concentrate was stored at -80°C until use.

2.2b PEG precipitation

The process for shellfish tissue homogenization was the same as the ultracentrifugation

method described previously. After stirring on ice for 1 hour, the homogenate was centrifuged at 5000 rpm for 40 minutes at 4°C. The supernatant was collected and adjusted the pH value to 7.0-7.5, followed by adding 8% PEG 6000 (EMD, CAS 25322-68-3, Made in Germany) (w/v). The PEG-viral solution was vortexed heavily until the PEG dissolved, and rotated on a Barnstead rotator (Dubuque, IOWA 52001, U.S.A) overnight at 4°C. The solution was then centrifuged at 10,000 x g for 1 hour at 4°C. The pellet was resuspended in 5 ml PBS and stored at -80°C until use.

2.3 Optimization of PEG method conditions

2.3a PEG concentration optimization

Comparative study of different PEG concentrations was conducted in this study to obtain the most optimal condition for viral exaction from shellfish. The spiked tissue homogenate which blending with 10 grams of shellfish tissue and 70 ml of TPB buffer (at 1:7 w/v) was transferred into a 100 ml beaker and stirred for 1 hour on ice, then centrifuged at 5,000 rpm for 40 min at 4°C. The supernatant was aliquoted into 5 portions and transferred into five 50 ml centrifugation tubes, PEG 6000 at concentrations: 5%, 8%, 10%, 12%, 15% (w/v) were added into the tubes, respectively, followed by rotating overnight at 4°C. After centrifugation at 10,000 x g for 1 hour at 4°C, the pellet was resuspended in 5 ml PBS for each concentration and stored at -80°C until use.

2.3b Tissue/TPB buffer dilution ratio optimization

4 tubes of 5 grams shellfish tissue were diluted at ratios of 1:1, 1:2, 1:5, 1:7 (w/v) with 10% tryptose phosphate broth/0.05M glycine buffer (pH9.5), respectively, followed by the homogenizing process and 1 ml of Poliovirus 1 stock with viral titer of 1 x 10^5 to 10^6 FPU/ml was spiked into each tube. The homogenate was then applied to PEG precipitation method.

2.4 Samples pretreatment

All samples were pretreated with Antibiotic Incubation Medium (2 mM P/S/G, 25ug/ml Amp B, and 500ug/ml Gentamicin, with 1x MEM medium) (AIM) at 1:2 at 4°C for 2-4 hours before plaque assay.

2.5 Cell culture-based infectivity assay-plaque assay

All the comparisons of different methods and conditions were applied to plaque assay to evaluate the viral titer. The cell preparation and plaque assay process were the same as described previously.

3. Results

3.1 Comparison of different virus concentration methods

Table 5 shows the comparison of the recovery rate of Poliovirus 1 concentrated from shellfish tissue conducted by two different methods, i.e., PEG precipitation method and ultracentrifugation method. The recovery rate for PEG precipitation method and ultracentrifugation method are found to be 77.84% and 51.55%, respectively, which indicates that PEG precipitation method is significant better than ultracentrifugation method ($p<0.001$).

3.2 Optimization of PEG method conditions

3.2a PEG concentration

Table 6 and Figure 7 show the comparison of the recovery rate among different PEG concentrations (5%, 8%, 10%, 12% and 15%) (w/v). It is displayed that the recovery rate of the infectious viral concentration from the shellfish tissue increases as the increase of concentration of PEG 6000 and it reaches to the highest point of 81.76% when the concentration of PRG 6000 is equal to 12% (w/v). Moreover, there is no significant difference between the recovery efficiency of 10% and 12% of PEG 6000 ($p = 0.991$) at which the recovery rate is much better than that of other concentrations ($p = 0.001$). Therefore, 10% of PEG 6000 is applied in this study to minimum the potential cytotoxicity caused by PEG.

3.2b Tissue/TPB buffer dilution ratio optimization

Table 7 and Figure 8 show the comparison of the viral recovery rate performed by PEG precipitation method with different dilution ratios of tissue with TPB buffer. It is found that the highest recovery rate of 86% appears when the shellfish tissue was diluted with TPB buffer at 1:2 (w/v) which might recover significant more infectious Poliovirus 1 comparing with other ratios ($p = 0.009$).

Table 5. Comparison of two different extraction methods for infectious Poliovirus 1 from shellfish tissue.

	Shellfish Extraction Methods		
Methods	**Mean Titer (PFU/ml)**	**Virus Input (PFU/ml)**	**Recovery Rate (%)**
PEG 6000	1.30×10^6	1.67×10^6	77.84±10.71
Ultracentrifugation	1.72×10^6	3.33×10^6	51.55±6.4

Table 6. Comparison study of different PEG 6000 concentrations.

PEG concentration (%) (w/v)	**Viral Input (FPU/ml)**	**Mean Titer of recovery virus(FPU/ml)**	**Recovery Rate (%)**
5	2.65×10^6	1.40×10^6	52.83±7.55
8	2.65×10^6	1.53×10^6	57.86±5.76
10	2.65×10^6	1.87×10^6	79.43±6.50
12	2.65×10^6	1.73×10^6	81.76±2.72
15	2.65×10^6	0.87×10^6	65.41±8.71

Table 7. The comparison study of different dilution ratios of shellfish tissue and TPB buffer.

Dilution Ratio (w/v)	Viral input (FPU/ml)	Mean Titer of recovery virus (FPU/ml)	Recovery Rate (%)
1:1	5.78×10^5	3.00×10^5	51.92±8.65
1:2	5.78×10^5	5.00×10^5	86.54±15.38
1:5	5.78×10^5	2.77×10^5	47.88±11.13
1:7	5.78×10^5	2.93×10^5	50.77±8.71

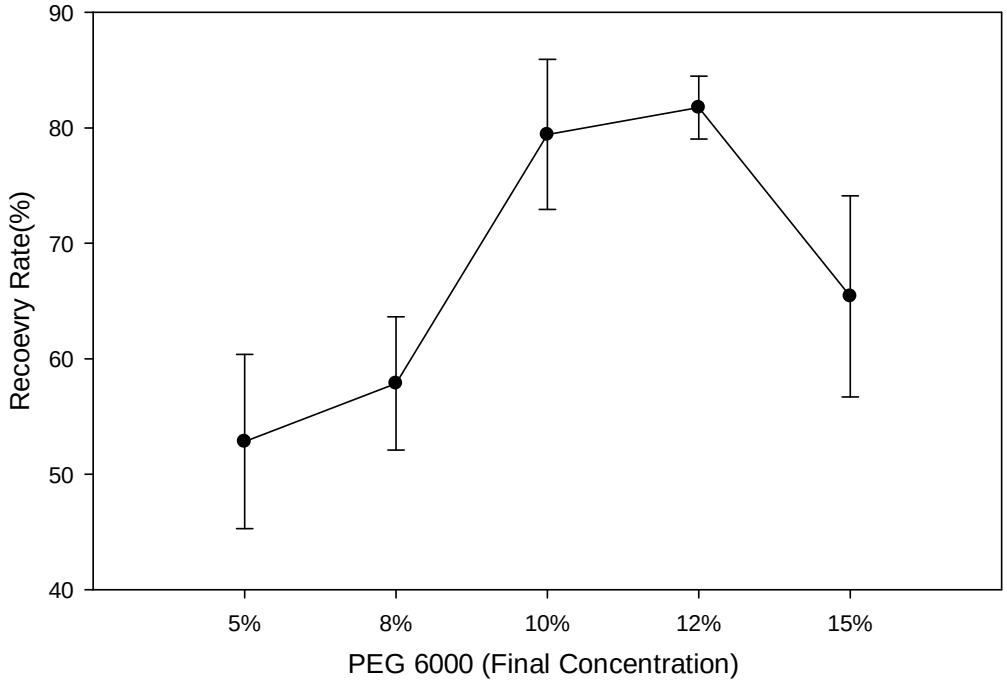

Figure 7. Graph of different PEG 6000 concentrations comparison.

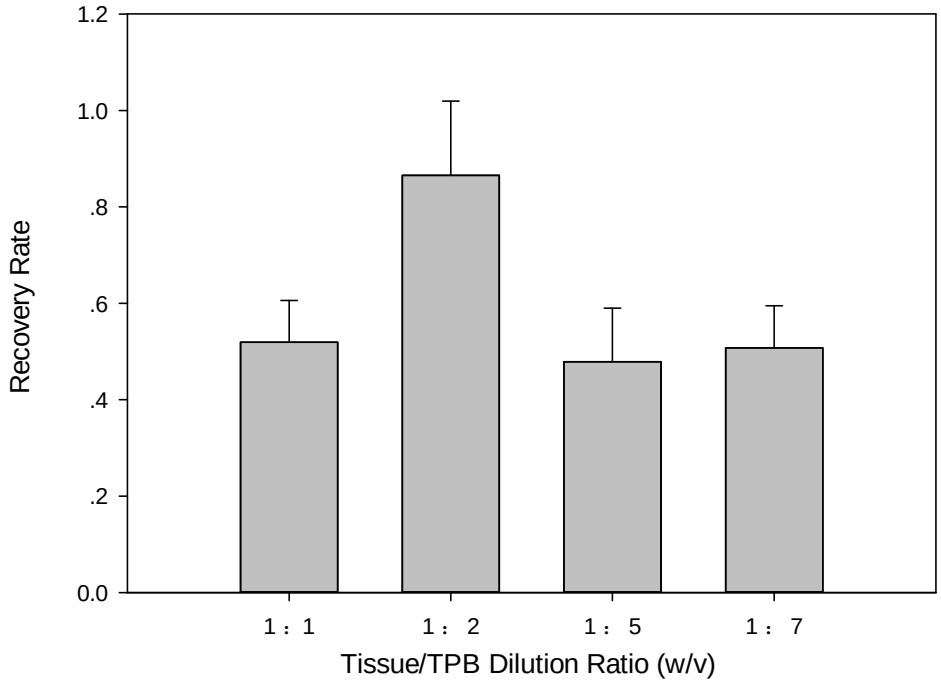

Figure 8. Graph of different dilution ratios of shellfish tissue and TPB buffer comparison.

4. Discussion

Previous studies reported that PEG precipitation is an effective method which has been used widely to concentrate infectious virus from tissue homogenates of shellfish. By using PEG precipitation, Zhou et al., obtained an average recovery rate of 89% and 91% for infectious rotavirus concentrating from oysters and clams, respectively in 1991. Jaykus et al, recovered 25-35% of Poliovirus 1 from oysters in 1996, while Lewis recovered 60-70% of Poliovirus 1 from pacific oysters and green-lipped mussel at the same year. In our study, 77% of infectious Poliovirus 1was successfully concentrated from the indigenous shellfish species (*isognomon spp.*), and the recovery rate has increased to 86% after optimizing the laboratorial conditions. It is observed that the recovery rates obtained by PEG precipitation method for infectious viral extraction from shellfish tissue vary from study to study, which might result from the different type of virus and shellfish species.

Gerba et al. in 1978 proposed a perspective that the shellfish tissue might contain cytotoxic components leading to a reduction of CPE occurrence in plaque assay. The shellfish species used in this study is *isognomon spp.*, belonging to the family Pteriidae. They are not suitable for commercial consumption due to their small size and meat weight (average 5cm in diameter and 1.5 grams of tissue per shellfish) comparing with other oysters and clams. Because of their small size, it is hard to separate the digestion system from the

whole body of shellfish. Therefore, instead of processing only the digestion system which was adopted in some other studies, the whole tissue of the indigenous shellfish was processed in our study which may possibly involve some impurities and bring potential harms to cell culture.

Additionally, because of its epifauna habitats, *isognomon spp.* usually lives on the surface, under the bottom or between slits of the big rocks close to the shallow seashores around the Oahu island, where it is easy for people to access. Compared to the shellfish used in other studies which were obtained from shellfish farms or laboratories, *isognomon spp.* live in a more complicated environment which may introduce more natural microorganism into their body. Based on Gerba's opinion, the recovery rate for infectious viral concentration in *isognomon spp.* might be limited by the impurities and unknown microorganism in their tissue.

Although the recovery rate could not reach as high as that of some studies reported, the method established in this study is still considered as an effective concentration method for infectious virus from the indigenous shellfish *isognomon spp.* in Hawaiian aquatic environment.

It is well known that PEG as a high-molecular-weight polymer could separate and precipitate small particles and the demand of final concentration of PEG in eluant would be associated with the size of target viruses. Our study showed that the most effective

concentration of PEG 6000 for participating Poliovirus 1 from *isognomon spp.* is 10 to 12%, and this result is consistent with the report conducted by Lewis et al., in 1988 that the optimized concentration of PEG for extracting HAV from shellfish was 12% due to the similar size between Poliovirus 1 and HAV.

As a rapid, efficient method for concentrating infectious virus from shellfish tissue, the PEG participation method optimized in this study would enhance the use of the indigenous shellfish species, i.e., *isognomon spp.* as a natural bio-indicaor for infectious virus detection in Hawaiian environmental water.

5. References

Asahina A. Y., Lu Y., Wu C., Fujioka R. S. and Loh P. C. (2009) **Potential Biosentinels of Human Waste in Marine Coastal Waters: Bioaccumulation of Human Noroviruses and Enteroviruses from Sewage-polluted Wasters by Indigenous Mollusks.** *Journal of Virological Methods* 158:46-50.

Comelli H. L., Rimstad E., Larsen S. and Myrmel M. (2008) **Detection of Norovirus genotype I.3b and II.4 in Bioaccumulated Blue Mussels Using Different Virus Recovery Methods.** *International Journal of Food Microbiology* 127: 53-59.

Croci L., Losio M. N., Suffredini E., Pavoni E., Pasquale S. D., Fallacara F. and Arcangeli G. (2007) **Assessment of Human Enteric Viruses in Shellfish from the Northern Adriatic Sea.** *International Journal of Food Microbiology* 144:252-257.

Dix A. B. and Jaykus L. A. (1998) **Virion Concentration Method for the Detection of Human Enteric Viruses in Extracs of Hard-shelled Clams.** *J. Food. Prot.* 61: 458-65.

Enriquez R., Frosner G. G., Hochstein-Mintzel V., Riedemann S. and Reonhardt G. (1992) **Accumulation and Persistence of Hepatitis A Virus in Mussels.** *J. Med. Virol.* 37: 174-179.

Formiga-Cruz M., Tofino-Quesada G., Bofill-Mas S., Lees D. N., Henshilwood K., Allard A. K., Conden-Hansson A. C., Hernroth B. E., Vantarakis A., Tsibouxi A., Papapetropoulou M., Furones M. D. and Girones R. (2002) **Distribution of Human Virus Contamination in Shellfish from Different Growing Areas in Greece, Spain, Sweden, and the United Kingdom.** App. *Environ. Microbiol. 2002:5990-5998.*

Gerba C. and Goyal S. M. (1978) **Detection and Occurrence of Enteric Viruses in Shellfish: A Review.** *Journal of Food Protection* Vol 41 (9) pp. 743-754.

Jaykus L., Leon R. D. and Sobsey M. D. (1996) **A Virion Concentration Method for Detection of Human Enteric Viruses in Oysters by PCR and Oligoprobe Hybridization.** *App. Environ. Microbiol.* 1996:2074-280.

Jothikumar N., Lowther J. A., Henshilwood K., Lees D. N., Hill V. R. and Vinje J. (2005) **Rapid and Sensitive Detection of Noroviruses by Using TaqMan-Based One-Step Reverse transcription-PCR Asays and Application to naturally Contaminated Shellfish Samples.** *App. Environ. Microbiol.* 2005:1870-1975.

Leggitt P. R. and jaykus L. A. (2000) **Detection Methods for Human Enteric Viruses in Representative Foods.** *J. Food. Prot.* 63:1738-44.

Lewis G. and Metcalf T. G. (1988) **Polyethylene Glycol Precipitation for Recovery of Pathogenic Viruses, Including hepatitis A Virus and Human Rotavirus, from Oyster, Water, and Sediment Samples.** *App. Environ. Microbiol.* 1988: 1983-1988.

Lewis G. D., Hough A., Green D. H. and Hay J. E. (1996) **Modification of the Polyethylene Glycol 6000 Precipitation Method for Recovering Human and Indicator Viruses from Oysters and Mussels.** *New Zealand Journal of Marine and Freshwater Research.* 30: 443-447.

Loisy F., Atmar R. L., guillon P., Cann P. L., Pommepuy M. and Guyader F. S. L. (2005) **Real-time PT-PCR for Norovirus Screening in Shellfish.** *Journal of Virolobical Methods* 123:1-7.

Luciana C., Nadia L. M., Giuseppe A., Tiziana P., Enrico P., Cristian M., Iole V. and Elisabetta S. (2009) **Survey of Enteric Virus Presence in Shellfish Products in Italy (1999-2008).** *ICMSS09-Nantes, France*-June2009.

Mesquita J. R., Vaz L., Cerqueira S., Castilho F., Santos R., Monteiro S., Manso C., Romalde J. L. and Nascimento M. S. J. (2011) **Norovirus, Hepatitis A Virus and Enterovirus Presence in Shellfish from High Quality Harvesting Areas in Portugal.** *Food Microbiology* 2011:1-6.

Metcalf T. G. and Stiles W. C. (1968) **Enteroviruses within an Estuarine Environment,** *Am. J. Epidemiol.* 88: 279-91.

Metcalf T. G., Moulton E. and Eckerson D. (1980) **Improved Method and Test Strategy for Recovery of Enteric Viruses from Shellfish.** *App. Environ. Microbiol.* 29:141-52.

Myrmel M., Berg E. M. M., Rimstad E. and Grinde B. (2004) **Detection of Enteric Viruses in Shellfish from the Norwegian Coast.** *App. Environ. Microbiol.* 2004:2678-2684.

Richard G. P. (1985) **Outbreaks of Shellfish-Associated Enteric Virus Illness in the United States: Requisite for Development of Viral Guidelines.** *J. Food. Project.* 48:815-823.

Sair A.I., Souza D.H. and Jaykus L.A. (2002) **Human Enteric Viruses as Causes of Food-borne Disease.** *Comprehensive reviews in food science and food safety.* Vol.1,2002.

Shieh Y. S. C., Monroe S.S., Fankhauser R. L., Langlois G.W., Burkhardt W. and Baric R.S. (2000) **Detection of Norwalk-like Virus in Shellfish Implicated in Illness.** *The Journal of Infectious Diseases.* 181: S360-6.

Sobsey M. D., Wallis C. and Melnick J. L. (1975) **Development of a Simple Method for Concentrating Enteroviruses from Oysters.** *Appl. Microbiol.* 29: 21-26.

Uhrbrand K., Myrmel M., Maunula L., Vainio K., Trebbien R., Norrung B. and Schultz A. C. (2010) **Evaluation of a Rapid Method for Recovery of Norovirus and Hepatitis A Virus from Oysters and Blue Mussels.** *Journal of Virological Methods* 169: 70-78.

Vajda B. P. (1978) **Concentration and Purification of Viruses and Bacteriophages with Polyethylene Glycol.** *Folia Microbiol.* 23: 88-96.

Vilarino M. L., Le Guyader F. S., Polo D., Schaeffer J., Krol J. and Romalde J. L. (2009) **Assessment of Human Enteric Viruses in Cultured and Wild Bivalve Molluscs.** *International Microbiology* 12:145-151.

Winn W. C. (1999) **Enterically Transmitted Hepatitis. Hepatitis A and E Viruses.** *Clin. Lab. Med.* 19: 661-673.

Zhou Y., Estes M. K., Jiang X. and Metcalf T. G. (1991) **Concentration and Detection of hepatitis A Virus and Rotavirus from Shellfish by Hybridization Tests.** *App. Environ. Mivrobiol.* 1991: 2963-2968.

Chapter 4

Evaluation of stability of Poliovirus 1 in different liquid environmental conditions

1. Introduction

Virus cannot grow without the hosts, however, they can persist in the environment for a certain period. Therefore, the transmission and survival of viruses are not only associated with their interaction with hosts, but also with environmental factors. Virus can survive without nutrients and keep infectious in different environmental conditions for a long time, which may result in many viral related outbreaks occurring without expectation (Rzezutka et al., 2004).

It is known that the longer time the virus could survive in the environment, the more chances they would obtain to transfer from one host to another (Rzezutka et al., 2004). Many environmental factors could influence the chances such as pH value, temperature, suspended solids, UV light and moisture (Fong and Lipp, 2005). It is necessary to understand the stability of enteric virus in environmental waters, also the influences of the environmental factors. This study will provide a clear notion of the potential health risk with pollutants, and is helpful to confirm the tools which are aimed to break the chain of viral transmission in order to decrease the chances for transmission (Rzezutka et al., 2004). This study is focused on the stability of Poliovirus 1 in different types of waters: PBS, seawater, freshwater and different percentage of raw sewage and sewage effluent. It is

anticipated that the study is able to give important information regarding viral stability in different aquatic conditions and facilitate our test to use enteric viruses as an indicator and assess contamination associated health risk.

2. Materials and Methods

2.1 Poliovirus 1 spiked process

500 ml of each liquid was contained in a 1 L conical flask and incubated in incubator(Thremo Electron corporation, REVCO, U.S.A.) at 20°C. Poliovirus 1 stock was spiked into each liquid with slightly shaking and stirring to reach a final titer of 1.4×10^5 FPU/ml. The top of the flasks were covered with tinfoil paper.

2.2 Different liquids comparison

Three different liquids were compared: seawater, freshwater and PBS. Seawater was obtained from Waikiki aquarium, Honolulu and was tested to confirm without infective poliovirus 1. Freshwater was obtained from tap water and also tested to confirm to be poliovirus-free.

2.3 Different percentages of raw and effluent sewage comparison

Raw sewage and sewage effluent which collected from Sand Island Wastewater Treatment Plant (SIWTT), Honolulu were added into an equal volume of 250 ml of sea water to reach a total volume of 500 ml. With the addition of 500 ml raw sewage and sewage effluent (Table 8), the stability of Poliovirus 1 was compared in these four liquids.

2.4 Water sample collection

1 ml of each liquid solution was sampled at specific time point: T =1, 3, 5, 7, 10, 15, 20, 25 and 30 (day), and store at -80°C until use. During sampling, the flask was slightly shook and stirred to mix the solution in order to increase the sampling accuracy. The samples were then centrifuged at 3,000 rpm for 5 minutes, and supernatant was collected.

2.5 Samples pretreatment

All samples were pretreated with Antibiotic Incubation Medium (2 mM P/S/G, 25ug/ml Amp B, and 500ug/ml Gentamicin, with 1x MEM medium) (AIM) at 1:2 at 4°C for 2-4 hours before plaque assay.

2.6 Cell culture-based infectivity assay-plaque assay

All the comparisons in different liquids were applied to plaque assay to evaluate the viral titer. The cell preparation and plaque assay process were the same as described previously.

Table 8. Components of different percentages of sewage waters.

	Liquid 1	**Liquid 2**	**Liquid 3**	**Liquid 4**
Sea water	0	50%	0	50%
Raw sewage	100%	50%	0	0
Effluent sewage	0	0	100%	50%

3. Results

3.1 Survival of Poliovirus 1 in different liquids comparison

Table 9 shows the stability of Poliovirus 1 in three different liquids environment, sea water, fresh water and PBS while Figure 9 display the plots of the survive of Poliovirus 1 in these three liquids which $Log_{10}(V_t/V_0)$ expresses the log relation between V_t and V_0 standing for the virus titer at time t and time zero, respectively. The rate constants (slopes) of the survival curves are considered as the corresponding decreases in $\log_{10}$ units per day (Ogorzaly et al., 2010). The time for a loss in titer of 90% of the input virus is designated as T_{90} (Fujioka et al., 1980). It is found that Poliovirus 1 is extraordinary stable in PBS which inactive only 1 $\log_{10}$ unit after 20 days. The stability of Poliovirus 1 in sea water is similar as that of PBS in the first 7 days. After this point, however, the viral amount in sea water falls dramatically and yields a T_{90} on Day 10 and no infectious virus could be detected after Day 15. In contrast, Poliovirus 1 is much more stable in freshwater than in seawater. It demonstrates that there is a slight decrease in fresh water after Day 7 and T_{90} in fresh water is obtained on Day 20.

3.2 Comparison of the survival of Poliovirus 1 in different percentages of raw and effluent sewage

Table 10 and Figure 10 show the results of the stability of Poliovirus 1 in different environments, i.e. 100% raw sewage, 100% sewage effluent, 50% raw sewage in sea water

and 50% sewage effluent in sea water. It is found that the behavior of the stability in 100% raw sewage is similar to that of 100% sewage effluent, and the same for 50% raw sewage and 50% sewage effluent. Moreover, the stability would not have a big difference at Day 6 among these four samples which indicating that the stability seems to be independent of the water environment on Day 6. Obviously, the log relationship as displayed in Figure 10 illustrates that the slope at the interval between Day 0 to Day 6 is much sharper than that of the interval greater than Day 6. Table 11 shows the rate constants splitting into two time intervals for these four samples and the overall slopes obtained by the linear regression. It is found that within the first six days, the constants for all the solutions are between -0.1 to -0.2, which indicates a 0.1 to 0.2 $\log_{10}$ unit decrease of viral titer per day. While after Day 6, the slopes become much gentle which are between -0.05 to -0.07 indicating that only 0.05 to 0.07 $\log_{10}$ is decreased every day in this time interval. It is also found that the Poliovirus 1 is more stable in 100% raw/effluent sewage than in 50% raw/effluent sewage when the overall slopes are compared. Extra five days were observed for Poliovirus 1 staying in 100% raw/effluent sewage than in 50% raw/effluent sewage which also demonstrates a better stability of Poliovirus in 100% raw/effluent sewage.

Table 9. Poliovirus 1 stability in different water conditions.

Time	Mean Titer in Sea Water (10^4 FPU/ml)	Mean Titer in Fresh Water (10^4FPU/ml)	Mean Titer in PBS (10^4FPU/ml)
Day 0	14.00±1.00	13.67±0.58	13.67±0.58
Day 1	9.00±1.73	7.00±2.00	8.00±1.00
Day 3	6.87±0.60	6.57±0.57	6.43±0.45
Day 5	6.53±0.42	6.50±0.44	6.10±0.26
Day 7	4.87±0.21	5.77±0.12	5.63±0.25
Day 10	0.03±0.01	2.30±0.17	4.37±0.38
Day 15	0	1.87±0.23	3.57±0.15
Day 20	0	1.07±0.29	2.63±0.15

Table 10. Stability of Poliovirus 1 in different percentages of sewage waters.

Time	Mean Titer in 100% Raw (10^4 FPU/ml)	Mean Titer in 100% Eff (10^4 FPU/ml)	Mean Titer in 50% Raw(10^4 FPU/ml)	Mean Titer in 50% Eff (10^4 FPU/ml)
Day 0	5.933±0.252	5.800±0.100	5.933±0.321	5.900±0.300
Day 1	1.200±0.10	0.967±0.351	3.967±0.208	2.967±0.379
Day 2	1.100±0.173	0.600±0.200	3.533±0.416	2.467±0.231
Day 3	0.553±0.038	0.357±0.060	2.433±0.153	2.233±0.306
Day 4	0.550±0.036	0.420±0.020	2.300±0.265	2.167±0.153
Day 5	0.480±0.026	0.400±0.010	0.700±0.157	0.863±0.111
Day 6	0.433±0.040	0.403±0.035	0.503±0.114	0.747±0.070
Day7	0.397±0.025	0.403±0.025	0.310±0.036	0.380±0.026
Day10	0.380±0.026	0.400±0.010	0.103±0.023	0.153±0.021
Day15	0.300±0.036	0.380±0.020	0.063±0.006	0.103±0.021
Day20	0.173±0.021	0.167±0.015	0.03±0.000	0.037±0.012
Day25	0.050±0.010	0.033±0.015	0	0
Day30	0	0	0	0

Table 11. Rate constants of the relation between Log_{10} (V_t/V_0) and time in different percentages of sewage water.

Sample	From 0 to 6 day	From 6 to 30 day	Overall
100% Raw	**-0.161**	**-0.054**	-0.059
100% Eff	**-0.157**	**-0.058**	-0.056
50% Raw	**-0.175**	**-0.058**	-0.090
50% Eff	**-0.136**	**-0.065**	-0.087

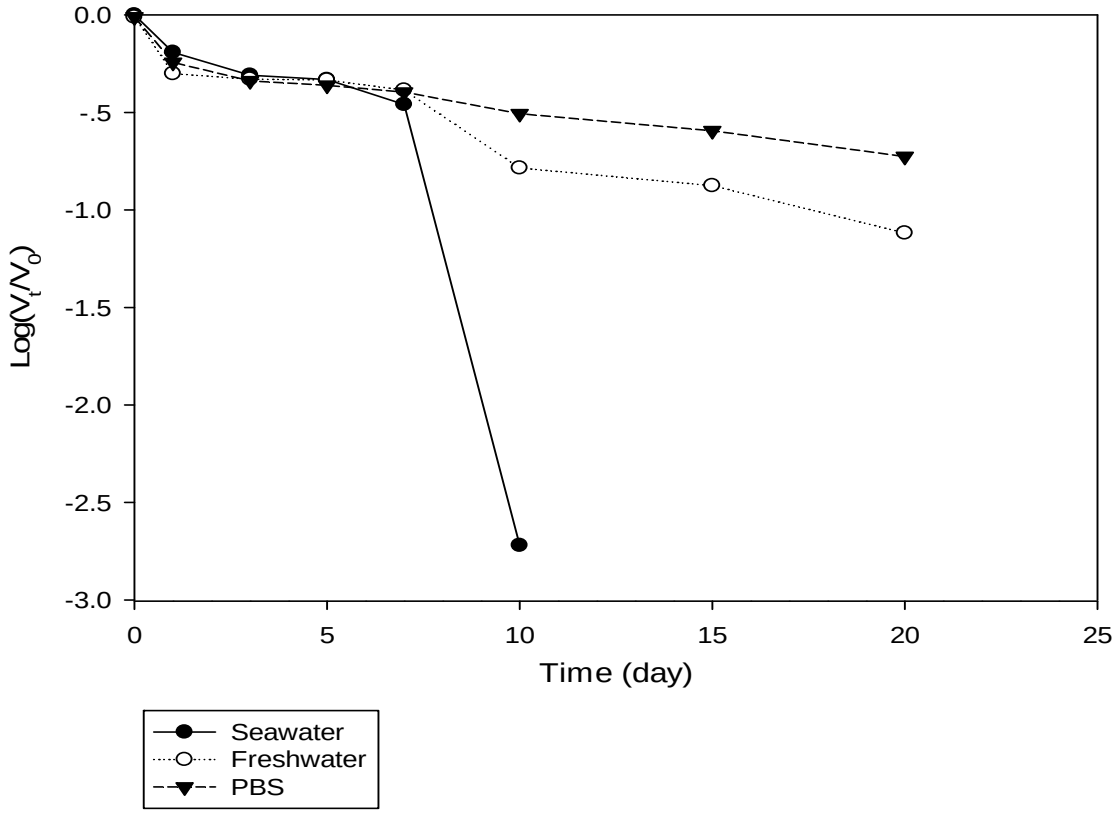

Figure 9. The survival functions of Poliovirus 1 in different water conditions.

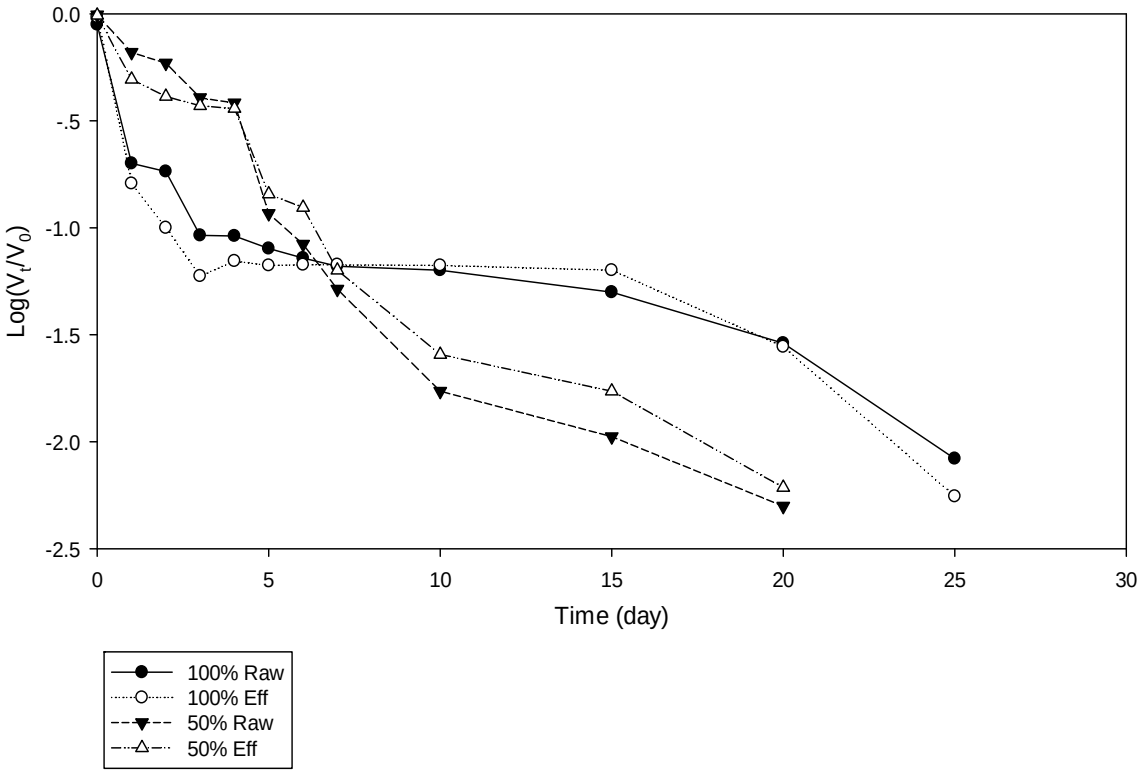

Figure 10. The survival function of Poliovirus 1 in different percentages of sewage waters.

4. Discussion

In this study, the stability of Poliovirus 1 in different aquatic environments has been determined. Although the viral stability might be affected by other environmental factors such as temperature, sea water source and virus types, it is concluded that virus may survive for a shorter time in sea water than that of fresh water and PBS, which is the same as previous studies (Bosch, 1995; Callahan et al., 1995; Stoner and Merkel, 2004). It is found in our study that Poliovirus 1 is the least stable in sea water that reduces 3 $\log_{10}$ in 10 days, which consistent with Callahan et al.'s study published in 1995 showing a 4 $\log_{10}$ reduction of Poliovirus 1in one week in sea water. The reason for the instability in sea water was reported that a variety of potential antiviral microorganisms in sea water might inactivate virus particles by breaking the viral proteins and thus digesting the exposed viral RNA (Abad et al., 1994; Bosch et al., 1993; Fujioka et al., 1980; Rzezutka et al., 2004).

In this study, stability of Poliovirus 1 was also investigated in 100% raw sewage, 100% sewage effluent, 50% raw sewage in sea water and 50% sewage effluent in sea water. All experimental results show the same behavior that the decrease rate of Poliovirus 1 stability is 2 to 3 times higher in the interval from Day 0 to Day 6 than that of the interval after Day 6, which indicates that Poliovirus 1 has a prominent reduction (1 $\log_{10}$) in the first 6 days, but thereafter a rapid gentle inactivation occurred with an approximately decrease rate of 0.06 $\log_{10}$ per day in the four solutions.

The overall slopes for the stability of Poliovirus 1 in different percentages of sewage environments illustrate that the decrease rate in 50% raw/effluent sewage is significantly higher than that of 100% raw/effluent sewage. This result could be explained by the following two reasons:

First, there are many naturally or artificially occurring sediments in both raw sewage and the sewage effluent which may have a protective effect for virus. It was shown that infectious virus could settle in the bottom's sediments of water for days to months, especially when it attaches with the solids in sewage (Stoner and Merkel, 2004). A study in 2003 reported that viable virus could survival in sediments up for 17 months (Griffin et al., 2003), while another study demonstrated that Poliovirus 1 survived for one-week longer in seawater with sediments than in seawater without sediments, and explained it as sediment could trap virus in surface openings or stabilizing them by electrostatic forces (Smith et al., 1978). In our study, the shaking of the conical flask during water sampling process which is aimed to minimum the experimental bias caused by viral settlement on sediments would release virus back from sediments to the water, thus, to obtain an accurate result on Poliovirus 1stability in sewage.

Second, the sea water mixed with the 50% raw and effluent sewage would also contribute to the inactivation of virus. It was illustrated that the virus-inactivating agents of a microbiological nature may exist in both clean and sewage-polluted seawater (Fujioka et

al., 1980; Rzezutka et al., 2004). In a summary, this study demonstrated that sea water could provide a steady inactive function to infectious virus, on the other hand, raw sewage and sewage effluent could be considered as viral carriers during the transmission process of infectious viruses.

5. References

Abad, F. X., Pinto R. and Bosch A. (1994) **Survival of Enteric Virus on Environmental Fomites.** *App. Environ. Microbiol.* 60: 3704-3710.

Bosch A. (1995) **The Survival of Enteric Viruses in the Water Environment.** *Microbiol. Sem.* 11:393-396.

Callahan K. M., Taylor D. J. and Sobsey M. (1995) **Comparative Survival of Hepatitis A Virus, Poliovirus and Indicator Viruses in Geographically Diverse Saewaters.** *Water. Sci. Rechnol.* 31:189-193.

Fong T.T. and Lipp E. K. (2005) **Enteric Viruses of Human and Animals in Aquatic Environments: Health Risks, Detection, and Potential Water Quality Assessment Tools.** *Microbiology and molecular biology review*, June 2005, p. 357-371.

Fujioka R. S., Loh P. C. and Lau L. S. (1980) **Survival of Human Enteroviruses in the Hawaiian Ocean Environment: Evidence for Virus-Inactivating Microorganisms.** *App. Environ. Microbiol.* 1980:1105-1110.

Griffin D.W., Donaldson K. A., Paul J. H. and Rose J. B. (2003) **Pathogenic Human Viruses in Coastal Waters.** *Clinical Microbiology Reviews*, Jan. 2003., p. 129-143.

Loh P. C. and Fujioka R. S. (1980) **Viruses in Water: Their Detection, Survival and Disease Potential.** *In Environmental Survey Techniques for Coastal Water Assessment Conference Proceedings,* PP. 41-69. UNIHI-SEAGRANT-CR-80-01. Also University of Hawaii Sea Grant College Program, Honolulu.

Ogorzaly L., Bertrand I., Paris M., Maul A. and Gantzer C. (2010) **Occurrence, Survival, and Persistence of Human Adenoviruses and F-Specific RNA Phages in Raw Groundwater.** *App. Environ. Microbiol.* 2010:8019-8025.

Okoh A. I., Sibanda T. and Gusha S. S. (2010) **Inadequately Treated Wastewater as a Source of Human Enteric Viruses in the Environment.** *International journal of environmental research.* 2010,7,2620-2637.

Rzezutka A. and Cook N. (2004) **Survival of Human Enteric Viruses in the Environment and Food.** *FEMS microbiology Reviews* 28: 441-453.

Salo R. J. and Olive D. O. (1976) **Effect of Acid pH, Salts, and Temperature on the Infectivity and Physical Integrity of Enteroviruses.** *Archives of Virology.* 52: 269-282.

Smith E. M., Gerba C. P. and melnick J. L. (1978) **Role of Sediment in the Persistence of Enteroviruses in the Estuarine Environment.** *App. Environ. Microbiol.* 1978:685-689.

Stoner N. and Merkel M. (2004) **Swimming in Sewage.** *Natural Resources Defense Council.*

Chapter 5

Evaluation of the bioaccumulation function of Poliovirus 1 in shellfish tissue under laboratory conditions

1. Introduction

Shellfish as filter-feeding animals are able to filter gallons of water every day. Therefore, they are considered as a kind of vehicles of enteric viruses (Canzonier, 1971). As discussed previously, we have already established the protocols for effective concentration of infectious enteric virus in shellfish tissue. However, the accumulation and persistence of Poliovirus 1 in the indigenous shellfish, *isognomon spp.* are still not clear. Numerous studies have confirmed the function to accumulate pathogens in some species of shellfish (Gerba and Coyal, 1978; Sair et al., 2002; Richard, 1985). Therefore, it is very necessary to confirm and evaluate the accumulation ability of the indigenous shellfish species: *isognomon spp.* by comparing the viral titer in shellfish with that of surrounding water in order to facilitate the use of indigenous shellfish as a bio-indicator for Hawaiian environmental water quality monitoring. The study is also aimed to confirm the optimized methods established previously.

2. Materials and Methods

2.1 Shellfish sampling and Poliovirus 1 spiking

50 to 60 shellfish were sampled from Kahala Beach, Honolulu, and placed in a

50x26x27cm glass vat containing 11.6 L of sea water and 5.8 L of freshwater. Poliovirus 1 stock was spiked to reach a final titer of 1 x 10^4 FPU/ml. A small water filter (Whisper, Tetra Sales, U.S.A.) was used to keep water flow-running during the whole experiment.

2.2 Samples collection and process

Two to four shellfish (total 5 grams) and 1 ml of the sea water were collected at specific time points(T=1,2,3,4,5,6,7,9,11,15 day), the shellfish samples were processed using the PEG precipitation method we established and optimized previously. Water samples were centrifuged at 3,000 rpm for 5minutes and then the supernatant wastreated with AIM at 1:2 for 2-4 hours and stored at -80°C until use.

2.3 Cell culture-based infectivity assay-plaque assay

All the comparisons were applied to plaque assay to evaluate the viral titer. The cell preparation and plaque assay process were the same as described previously.

3. Results

Table 12 shows the viral titer of Poliovirus 1 as the increase of time in the shellfish *isognomon spp.* and its surrounding water, separately. While Figure 11 displays the plots of their relationships. The concentration of Poliovirus 1 in the surrounding water remains in a decreasing range and declined to zero in 11 days after the beginning of the experiment. Poliovirus 1 in the shellfish tissue could be initially detected on Day 2 in this experiment, rises sharply in the first 4 days, and then goes down to zero on Day 15.

In Figure 11, the concentration of Poliovirus 1 accumulated in the shellfish (*isognomon spp.*) is smaller at the beginning and then greater on Day 3 than that of its surrounding water. It is shown that the concentration of Poliovirus 1 in the shellfish tissue arrives at the maximum value on Day 4 as high as 3.27×10^4 FPU/ml which is 23 times greater than that of its surrounding water on the same day. Additionally, there is a small peak which the value is equal to 20% of the maximum peak observed in the depuration period.

Table 12. Survival of Poliovirus 1 in shellfish tissue and surrounding water.

Time	Mean Viral Titer in Water (10^3 FPU/ml)	Mean Viral Titer in Shellfish (10^3 FPU/ml)
Day 0	11.33±2.08	0
Day1	7.67±1.53	0
Day2	3.77±0.67	0.57±0.06
Day3	2.70±0.40	4.70±0.36
Day4	1.43±0.12	32.67±2.08
Day5	0.83±0.15	22.67±3.79
Day6	0.27±0.12	3.40±0.26
Day7	0.17±0.15	7.33±0.58
Day9	0.10±0.10	3.60±0.26
Day 11	0	1.13±0.21
Day15	0	0.53±0.29
Day19	0	0

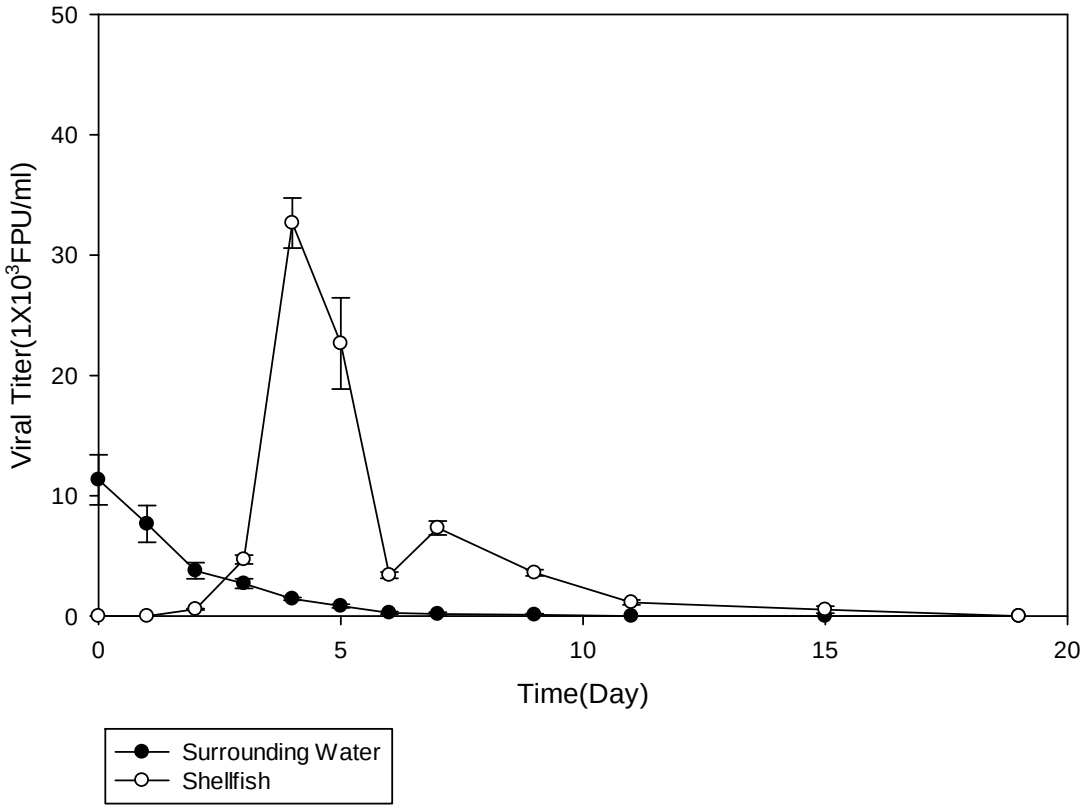

Figure 11. Graph of the stability of Poliovirus 1 in shellfish tissue and surrounding water.

4. Discussion

It was reported that shellfish could display a process of viral accumulation and elimination when it presents in the polluted water (Gerba and Coyal, 1978). Therefore, the indigenous shellfish *isognomon spp.* was applied to confirm the accumulation ability by spiking Poliovirus 1 into the surrounding water in this study. It was found that the concentration of Poliovirus 1 accumulated in the shellfish arrives at the value of 3.27 x 10^4 FPU/ml within 2 days and the uptake rate is 1.6 x 10^4 FPU/(ml·day) obtained by the linear regression.

The regression analysis of Poliovirus 1 concentrations in shellfish vs. time during the depuration phase of the data set according to first order kinetics give rate constant equal to –0.12/day. Thus, the decrease in Poliovirus 1 concentration during depuration can be represented by, $V_t = V_0\ 10^{(-0.12t)}$, where V_t is the Poliovirus 1 concentration (FPU/ml) in the shellfish at time t (day), V_0 is the Poliovirus 1 concentration in the shellfish at the beginning of the depuration period, and -0.12/day is the first order rate constant. According to this model, Poliovirus 1 concentration decreases to 50% of the maximum peak 2.49 days from the beginning of the depuration period which is 7.49 days after the initial time of this experiment.

Poliovirus 1 has performed a process of accumulation and elimination in the *isognomon spp.*'s tissue, however, the step-by-step progression of viruses from environmental sea

water into the shellfish tissue has not been followed precisely. To our best knowledge, it was confirmed that the increase virus in shellfish tissue is resulted from bio-accumulation because the multiplication of virus has not been demonstrated in shellfish tissue (Berg, 1978).

The accumulating rate of viral particles in shellfish is affected by many factors such as salinity, temperature, turbidity, shellfish species and also dependent on whether water was still water and flow-running water. In this study, the accumulation rate for the *isognomon spp.* was determined as 1.635×10^4 FPU/ (ml·day). During the depuration period, the decrease curve of shellfish *isognomon spp.* was described as the first order depuration which the $\log_{10}$ linear regression rate constant is given as -0.121 $\log_{10}$/day, and shellfish is able to retain Poliovirus 1 for 11 days. Obviously, Poliovirus 1 is more stable in shellfish than in its surrounding water since the half-loss of virus (1.63×10^4 FPU/ml) in shellfish was observed at Day 7.49 while the viral titer in water was only around 0.1×10^3 FPU/ml at the same time. Chou et al., demonstrated that the external particles will not enter the digestive system and are directly discharged to the environment when shellfish reaches the feeding capacity of them (Chou et al., 2005). Based on this assumption, it is possible that shellfish would start to accumulate virus again once the viral concentration in their tissue reduces to a certain level during the depuration period. Therefore, in this study, the small peak occurred during the depuration period is considered as the viral

re-adsorption of shellfish *isognomon spp.*. Actually, oscillations of bacteria/toxin content in shellfish tissue during the depuration period were also observed in some studies (Chou et al., 2005; Selegean et al., 2001). With the eliminating of the viral concentration in surrounding water, the oscillations trend to reduce and then disappear.

In a conclusion, this study demonstrated that shellfish *isognomon spp.* has a significant accumulation function of infectious Poliovirus with an accumulate rate of 1.635×10^4 FPU/ (ml·day), and Poliovirus 1 is proved more stable in shellfish than in its surrounding water. Since shellfish do not normally contain infectious virus inside their tissue, the presence of infectious virus in shellfish might indicate a recent environmental contamination, which makes the shellfish *isognomon spp.* a more sensitive and rapid indicator for infectious virus monitoring in Hawaiian aquatic environments.

5. References

Berg G. (1978) ***Indicators of Viruses in Water and Food.*** Publisher, Ann Arbor Science.

Canzonier W. J. (1971) **Accumulation and Elimination of Coliphage S-13 by the Hard Clam, Mercenaria Mercenaria.** *App. Environ. Microbiol.* 1971:1024-1031.

Chou H. N., Huang C. P. and Chen C. Y. (2005) **Accumulation and Depuration of Paralytic Shellfish Poisoning Toxins by Laboratory Cultured Purple Clam Hiatula Diphos Linnaeus.** *Toxicon* 46 (2005) 587-590.

Gerba C. and Coyal S. M. (1978) **Detection and Occurrence of Enteric Viruses in Shellfish: A Review.** *Journal of Food Protection* 41: 743-754.

Hedstrom C. E. and Lyke E. (1964) **An Experimental Study on Oysters as Virus Carriers.** *Am. J. Hyg.* 79:134-142.

Metcalf T. G. and Stiles W. C. (1965) **The Accumulation of Enteric Viruses by the Oyster Crassostrea Virginica.** *J. Infect. Dis.* 115:68-76.

Richard G. P. (1985) **Outbreaks of Shellfish-Associated Enteric Virus Illness in the United States: Requisite for Development of Viral Guidelines.** *J. Food. Project.* 48:815-823.

Sair A.I., Souza D.H. and Jaykus L.A. (2002) **Human Enteric Viruses as Causes of Food-borne Disease.** *Comprehensive reviews in food science and food safety.* Vol.1,2002.

Selegean J.P.W., Kusserow R., Patel R., Heidtke T. M. and Ram J. L. (2001) **Using Zebra Mussels to Monitor Escherichia coli in Environmental Waters.** *J. Environ. Qual.* 30:171-179.

Chapter 6

Conclusions

In this study, an alternative monitoring system applying direct detection of infectious enteric virus in recreational waters have been successfully established.

In order to extract infectious virus from sea water, the filtration-based concentration method by using negatively charged type HA membranes, the shaking elution method and cell culture technique for evaluating viral titer were combined to develop an effective laboratory protocol which could successfully extract more than 90% of infectious Poliovirus 1 from sea water.

PEG precipitation method was applied and optimized as an effective concentration method which could recover more than 77% of infectious Poliovirus 1 from the indigenous shellfish (*isognomon spp.*) tissue.

The stability of Poliovirus 1 in different aquatic environments and in shellfish was also determined, which demonstrated that Poliovirus 1 is the most unstable in sea water among all the liquids. Also Poliovirus 1 could survival for a longer time in 100% sewage water than that of 50% sewage water. However, there is no evidence illustrating a significant difference between Poliovirus 1 stability in raw sewage and sewage effluent. The indigenous shellfish (*isognomon spp.*) was confirmed to have the bio-accumulation

function for infectious Poliovirus 1, and also to retain the virus in their tissue for a longer time than that of its surrounding water.

The optimized infectious virus concentration protocols would be used for monitoring recreational water quality, which provide accurate and sensitive information about both qualitative and quantitative data for infectious virus presence and indicate potential contamination and public health risk of exposure. The stability studies have facilitated our test to use enteric viruses and shellfish as indicators to monitor the recreational water quality and assess the contamination associated health risk to the exposed swimmers.