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Concentration and detection of hepatitis A virus and its indicator from seawater

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CONCENTRATION AND DETECTION OF HEPATITIS A VIRUS AND ITS INDICATOR FROM SEAWATER

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I dedicate my dissertation to my beloved parents Shaoping Chen and Changxian Liu, and my husband Morgan André Maurice Cormier.

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Abstract

Hepatitis A virus (HAV) infection is the leading worldwide cause of acute viral hepatitis, and its outbreaks often occur from fecal contaminated shellfish. HAV is extremely stable in the environment and can survive 3~10 months in water. In addition, HAV can be bio-concentrated by shellfish by as much as 100 fold from fecal contaminated waters. Bacteriophage MS2 is used widely as a surrogate for HAV, and its presence has been proven to be a reliable indication of the presence of HAV. A rapid detection of viral contamination in water environments can prevent economic loss and can identify the source of contamination within a short time. However, the conventional methods for virus concentration and detection are often laborious, time consuming, and subject to clogging. Furthermore, most methods require a secondary concentration step to further reduce the final volume of samples. Hence the objective of this study was to develop a simple, rapid and inexpensive virus concentration method in aid of rapid detection. The use of granular activated carbon and zeolite were investigated. In the method, high levels of viruses (HAV or MS2) were inoculated into artificial seawater and concentrated by the adsorbents. The viruses were then eluted with protein denaturant and detected via real-time PCR (qPCR). While both adsorbents were more efficient in seawater than in fresh water, and were able to adsorb 6 logs of viruses from seawater, zeolite was able to adsorb ~99% of the viruses in less than 5 min at room temperature, and the entire concentration and detection can be done in approximately 2 h. Compared to existing methods, this method eliminated the need for a secondary concentration step, as well as the necessity to modify the pH or salinity of the seawater during concentration. The virus concentration method using activated carbon or zeolite could be a useful addition to the available methods for virus detection in seawater.

Chapter 1. Introduction

Enteric viruses such as hepatitis A virus (HAV) are responsible for a large proportion of food and water-borne illnesses. These viruses are transmitted to humans via the fecal-oral route, usually from contaminated water or foods such as raw shellfish (Ajelli and Merler, 2009; Bosch, 1998; Dubois et al., 2002; Kingsley et al., 2002; Richards, 2001; Roldán et al., 2013; Sánchez, 2013; Woods, 2013). HAV infection is the leading worldwide cause of acute viral hepatitis, and its outbreaks have occurred among consumers of shellfish harvested from fecal polluted waters.

HAV is resistant to low pH (< pH 1) and to heating, surviving 1 h at 60ºC (Lemon, 1992). It appears to be extremely stable in the environment, with only a 100-fold decline in infectivity over 4 weeks at room temperature, and 3~10 months in water (Hollinger and Ticehurst, 1996; Koopmans et al., 2002). HAV appears to be relatively resistant to free chlorine, especially when the virus is associated with organic matter.

Viral contaminated shellfish can be a public health concern, as shellfish are filter feeders that can actively bio-concentrate HAV as much as 100 fold from fecal contaminated waters (Le Guyader et al., 2012; McLeod et al., 2009; Nappier et al., 2008; Wang et al., 2008). Even point source discharge of human waste from commercial and recreational vessels can result in viral contamination of approved shellfish beds without observation of increase in fecal coliform in marine water samples. The National Shellfish Sanitation Program recommends immediate closure of shellfish growing area when enteric viral pathogens are suspected (National Shellfish Sanitation Program, 2011).

Traditional indicator organisms like fecal coliform behave as a good indicator for enteric bacteria but a poor indicator for enteric viruses. In contrast, male-specific bacteriophages have been proposed as indicators of enteric viruses, and clear correlations have been observed between

male-specific bacteriophages and enteric viruses because their morphology and survival characteristic closely resemble HAV and other enteric viruses (Blaise-Boisseau et al., 2010; DePaola et al., 2010; Meschke et al., 2003; Serracca et al., 2010). Several scientific studies have confirmed that, for monitoring purposes, phages are reliable indicators of the possible presence of human enteric viruses in seafood and seawater. As a group I male-specific RNA coliphage that infects *Escherichia coli* (Nappier et al., 2008), MS2 is used widely as a surrogate for enteric viruses, and its presence has been proven to be a reliable indication of the presence of HAV. It can survive for a relatively long time in the environment, similar to that of HAV (Allwood et al., 2003; Huertas et al., 2003; Katz and Margolin, 2007; Shin et al., 2003; Tree et al., 2003). Unlike most human pathogenic enteric viruses which cannot be cultured, MS2 can be detected by culture assays and molecular biology assays.

A rapid detection of viral contamination in water environments can prevent economic loss from closure of recreation beach and shellfish harvesting sites, and can identify the source of contamination within a short time. Unfortunately, numbers of viruses and phages in contaminated food or water are usually too low for detection; therefore, the development of an efficient concentration method is critical. Absorption/elution and ultrafiltration methods are frequently employed to concentrate viruses from large volumes of water. Absorption/elution methods take advantage of the principle that viruses are negatively charged at alkaline pH. Ultrafiltration methods employ filters with small pore sizes to allow viruses to be retained within the circulating sample. Both methods need expensive and complex machinery, and are subject to clogging of the filters, which slows down flow rate and reduces recover efficiency of the viruses; in addition, a second concentration step is usually needed to further reduce the volume of the eluate (Gibbons et al., 2010; Grant et al., 2011; Karim et al., 2009; Kreißel et al., 2012; Rigotto et al., 2009).

In an effort to solve many of the disadvantages associated with these methods and come up with one that would reduce the time and cost of virus concentration, the use of activated carbon and zeolite were investigated. Activated carbon is commonly used in water reclamation for organic matter removal and its large surface area (500~1400 m^2/g) grants sufficient adsorption (Cheremisinoff and Ellerbusch, 1978; Cormier et al., 2012; Le Cloirec., 1997; Takeuchi et al., 1997; Ternes et al., 2002). Zeolite is a unique micro-porous mineral rock and is known as an ion-exchange mineral for its ability to attract and bond certain ions (Booker et al., 1996; Perić, 2004; Wojcik, 2001). As nano-scale organic matters with negative charge, HAV and MS2 could be concentrated with zeolite or activated carbon and detected with real-time PCR.

The overall goal of this project was to develop a concentration method that aids the rapid detection of HAV in shellfish growing areas, using MS2 as a surrogate. This was a two-stage process, which entailed sampling large volumes of seawater (10-20 L) and concentrating the viruses, followed by detection using real-time PCR (qPCR). The specific objectives were:

1) Optimization of cell infectivity assays of HAV and MS2.

2) Optimization of qPCR for detection of HAV and MS2.

3) Optimization of virus concentration with activated carbon and zeolite.

4) Evaluation of different parameters of seawater on the efficiency of virus concentration.

5) Development of large scale (10 L) concentration of HAV from seawater.

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Chapter 2. Literature review

2.1 General information on hepatitis A virus

Hepatitis A virus (HAV) is the sole member of the *Hepatovirus* genus within the family *Picornaviridae* (Graff et al., 1994). It is a small, non-enveloped spherical virus, measuring between 27 and 32 nm in diameter (Fig 2.1). HAV enters the body via ingestion, multiplies in the intestine and spreads to the liver through blood stream. The incubation period for HAV is about 4 weeks (Sinclair et al., 2009). HAV is stable in the environment and particularly resistant to disinfectants, heating pressure, and low pH (Lemon, 1992).

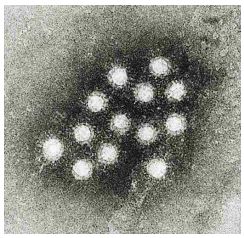

Fig. 2.1 Hepatitis A virus (Centers for Disease Control and Prevention, 1976)

2.2 Cultural and biochemical characteristics of HAV

HAV is a Baltimore class IV virus that contains a single positive stranded RNA genome of approximately 7.5 kb. Positive sense viral RNA is similar to mRNA and thus can be immediately translated by the host cell. The purified RNA of a positive sense virus can directly cause infection though less infectious than the whole virus particle (Carter and Sanders, 2007). The genome of HAV encodes a large polyprotein, which is cleaved to four structural and seven non-structural proteins by proteinases encoded in and around the 3C region. The cleavages occur before translation is complete and are carried out by virally coded proteases (Fig 2.2).

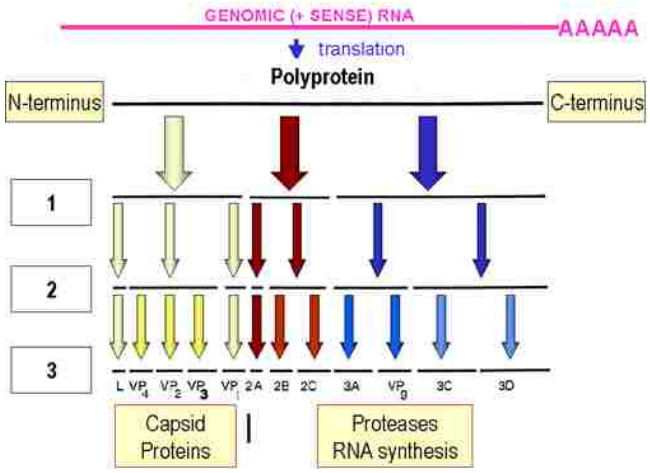

Fig. 2.2 Processing of polyprotein of HAV (Schaechter et al., 1993)

Replication efficiency seems to be controlled by amino acid substitutions in the 2B and 2C regions (Najarian et al., 1985; Probst et al., 1998; Yokosuka, 2000). The large open reading frame present in the genome of HAV can be divided into three functional regions, termed P1, P2 and P3. The P1 genomic region encodes the capsid polypeptides (VP1, VP2, VP3 and a putative VP4), and the P2 and P3 regions encode the non-structural polypeptides necessary for virus replication (Rueckert and Wimmer, 1984).

The 5' end of its genome RNA is covalently attached to a genome-linked viral protein (VPg) through a phosphodiester bond between tyrosine and the terminal uridylyl residue of the RNA (Fig 2.3). The Vpg acts as a replicase and a protease and has a molecular weight of 2500 Dalton with an isoelectric point of 7.15 (Flanegan et al., 1977; Lee et al., 1977; Weitz et al., 1986). HAV is an icosahedral particle composed of four major polypeptides cleaved from a large polypeptide precursor, and its capsid proteins associate to form a protomer, five protomers form a pentamer, and 12 pentamers comprise a viral capsid. Three of the structural capsid proteins found in the mature particles (VP1, VP2, VP3) are approximately 25-35 kDa, and analysis of their sequence predicts the presence of both β-sheet and α-helical regions. The surface-exposed regions of VP1 and VP3 contain the major antigenic epitope and the major binding site for neutralizing

antibody (Coulepis et al., 1982; Gauss-Müller et al., 1986; Putnak and Phillips, 1981; Siegl et al., 1981; Volkin et al., 1997).

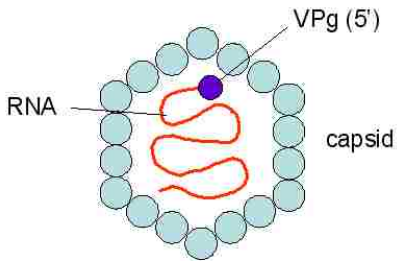

Fig. 2.3 Structural illustration of HAV (Murray et al., 2005)

2.3 Life Cycle of HAV

The transmission of HAV is generally due to the ingestion of material contaminated with feces containing HAV. However, the pathological sequence of events that begins with entry of the virus via the gastrointestinal tract and ultimately results in hepatitis is not well understood. In recent years, HAV antigen was found within cells of the small intestine (Blank et al., 2000). In two different animal model studies using inoculated primates, HAV was found present in the gut prior to its detection within hepatocytes, suggesting that small intestinal epithelial cells serve as a primary site of replication for HAV (Asher et al., 1995; Karayiannis et al., 1986). Blank and others (2000) identified HAV replication within Caco-2 cells, which most closely resemble epithelial cells of the small intestinal villi and crypts (Hidalgo et al., 1989; Quaroni, 1986). This finding was consistent with the observation that intestinal epithelial cells are infected by virus present within the lumen of the gastrointestinal tract (Asher et al., 1995; Karayiannis et al., 1986). However, the restricted basolateral release of HAV Caco-2 cells suggests that epithelial cell infection is unlikely to result in penetration of the virus beyond the gastrointestinal epithelium. Thus, HAV invasion of deeper tissues, a requirement for its eventual passage to the liver, may be dependent upon alternate mechanisms (Blank et al., 2000).

The major cell type which supports HAV replication is the hepatocyte, is also highly polarized and of epithelial origin (Crawford, 1996, Hubbard et al., 1994). The apical surface of the hepatocyte forms a well demarcated groove which encircles the cell and provides access to the biliary canaliculi. During acute hepatitis A, components of bile and HAV are secreted from the liver into the feces (Crawford, 1996; Huang et al., 1979; Schulman et al., 1976). The extended basilar surface of the hepatocyte is exposed to the perisinusoidal space and through it to the venous sinusoids, via which HAV is likely to reach the liver during the early stages of the infection.

HAV binds to a receptor that is found on the surface of hepatocytes and a few other cells. HAV cellular receptor 1 (havcr-1) has an ectodomain that contains an N-terminal cysteine-rich immunoglobulin-like region, followed by a mucin-like region that extends the immunoglobulin-like region well above the cell surface. The immunoglobulin-like region is required for binding of HAV. The virus spends its entire life in the cytoplasm where it replicates using a virus-encoded RNA-dependent RNA polymerase. The viral RNA functions as an mRNA but does not have the methylated cap structure typical of eukaryotic mRNAs, instead, it has a an internal ribosome entry site (IRES) which enables ribosomes to bind without having to recognize a 5' methylated cap structure (Fig 2.4). HAV can interfere with host cell methylated cap recognition, and since most host cell translation is cap-dependent, host protein synthesis is inhibited but not viral protein synthesis (Murray et al., 2005).

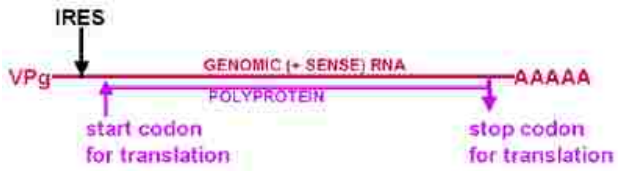

Fig. 2.4 Internal ribosome entry site of HAV (Murray et al., 2005)

The replication of HAV genome starts by viral RNA polymerase copying plus sense genomic RNA into complementary minus sense RNA using VPg as a primer for RNA synthesis.

New minus sense strands serve as template for new plus sense strands (Fig 2.5). When sufficient plus-sense progeny RNA and virion proteins have accumulated, assembly begins. Particles assemble with VPg-RNA inside and 3 proteins in the capsid (VP0, VP1 and VP3). VP0 is then cleaved to VP2 and VP4 as the virions mature and these mature virions are released following cell lysis (Murray et al., 2005).

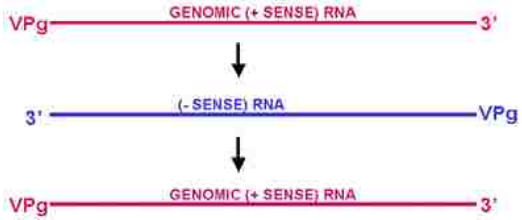

Fig. 2.5 Replication of HAV (Murray et al., 2005)

2.4 Genotyping of HAV

There is only a single serotype of HAV, since antibodies elicited by either natural or experimental infection do not distinguish between individual strains of HAV (Gellis et al., 1945; Lemon and Binn, 1983; Neefe et al., 1946; Provost et al., 1973; Rakela et al., 1976). Due to high degree of antigenic conservation among human HAV strains, infection with HAV is likely to confer life-long immunity that protects against subsequent symptomatic reinfection.

HAV was originally divided into seven major genotypes from molecular methods based on the putative VP1⁄2A junction. Each genotype is defined as a group of viruses having nucleotide sequences which differ from each other at no more than 15% of base positions (Jansen et al., 1991). Genotype I, II, III and VII are associated with human disease, whereas the remaining three genotypes (IV, V and VI) each include a single unique simian HAV strain. However, genotype VII has recently been reclassified as a sub-genotype of genotype II, resulting in only six major genotypes of HAV being classified (Costa-Mattioli et al., 2002; Lu et al., 2004). Genotype III is unique in that it includes strains that have been recovered from both human and non-human

primate sources. Genotype I includes 82 of the 104 (80%) human HAV strains studied, and is divided into two sub-genotypes (IA and IB) differing from each other at approximately 7.5 % of base positions (Jansen et al., 1991).

Sub-genotype IA comprises the majority of the human strains studied (69 of 104, 67%), and includes strains found worldwide. It constitutes the major virus population in North and South America, China, Japan, the former U.S.S.R. and Thailand. Three geographically related clusters of viruses with closely related sequences are found in this genotype. These include one group of strains from the U.S.A., another from Japan and a third group recovered both in Japan and China. The remaining strains within the IA sub-genotype appear to be more randomly dispersed with respect to geographical origin. Sub-genotype IB contains strains from Jordan, North Africa, Australia, Europe, Japan and South America. The majority of these strains were recovered from locations near the Mediterranean. Most of the remaining human HAV strains segregate into a single genotype that can also be divided into two sub-genotypes, IIIA and IIIB. The prototype virus strain of the IIIA sub-genotype, PA21, was originally isolated from recently captured Panamanian owl monkeys (Robertson et al., 1992).

2.5 Epidemiology of HAV infection

Infection with hepatitis A virus (HAV) is the leading cause of clinically apparent viral hepatitis in the United States. HAV infection is generally self-limiting and is primarily transmitted via the fecal-oral route through contact with an infected person (Centers for Disease Control and Prevention, 2011; Mast and Alter, 1993). Symptoms of HAV infection include nausea, anorexia, fever, malaise, abdominal pain, jaundice, dark urine, or elevated serum alanine aminotransferase (ALT) >200 IU/L. Most recent hepatitis A incident infections involve food handlers. Historically, acute hepatitis A rates vary cyclically, with nationwide increases every 10-15 years. The last peak

was in 1995 (Centers for Disease Control and Prevention, 2010); since that time, rates of hepatitis A have steadily declined by 95% (Fig 2.6).

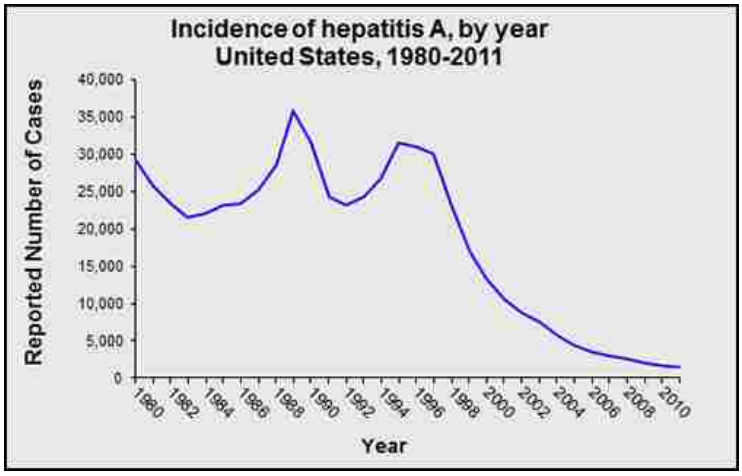

Fig. 2.6 Incidence of HAV (Centers for Disease Control and Prevention, 2012)

The incidence of HAV infection varies considerably among and within countries. In much of the developing world, where HAV infection is endemic, the majority of people are infected in early childhood and virtually all adults are immune. In the developed countries, however, HAV infections have become less common as a result of increased standards of living. Only few people are infected in early childhood, and the majority of adults thus remain susceptible to infection, which may lead to the occurrence of outbreaks of hepatitis A among the general population (Sanchez et al., 2007).

Person-to-person transmission through the fecal-oral route is the primary means of HAV transmission in the United States (Fiore, 2004; Hollinger and Emerson, 2001). Most infections result from close personal contact with an infected household member or sex partner. Common-source outbreaks and sporadic cases also can occur from exposure to fecal contaminated food or water. Uncooked HAV-contaminated foods have been recognized as a source of outbreaks. Cooked foods also can transmit HAV if the temperature during food preparation is inadequate to kill the virus or if food is contaminated after cooking, as occurs in outbreaks associated with

infected food handlers. Waterborne outbreaks are infrequent in developed countries with well-maintained sanitation and water supplies (Centers for Disease Control and Prevention, 2013). The infective dose of HAV for humans is extremely low; even one infective HAV is sufficient to cause hepatitis A infection in humans. Outbreaks of hepatitis A due to consumption of raw or inadequately cooked shellfish harvested from sewage-polluted waters have been documented (Desenclos et al., 1991; Halliday et al., 1991). HAV is often detected in shellfish, even when the levels of fecal coliforms are low.

HAV is one of the few enteric viruses for which conclusive evidence of waterborne transmission exists, and common source outbreaks have been attributed to fecal contaminated food and drinking water (Rao and Melnick, 1986). A causal association between hepatitis A and the use of fecal contaminated water for recreation purposes has also been proven (Mahoney et al., 1992). The presence of viable HAV in the river and dam water is therefore a potential infection hazard for the lower and higher socioeconomic communities using these waters for domestic or recreational purposes (Taylor et al., 2001).

2.6 Seasonal occurrence of HAV

The observation of the presence of HAV in the two separate study periods imply a greater likelihood of the shellfish harboring HAV in autumn and winter, coinciding with the seasons that shellfish are among the most popular dining choices locally (Lee et al., 1999). HAV was detected in 35.3% of the river and 37.3% of the dam water samples in South Africa in a study performed by Taylor and others (2001). They also discovered that HAV was present throughout the year with a seasonal peak being evident in both the river and the dam water in early spring, while seasonal peaks of hepatitis A infection in autumn and winter have been noted in some temperate countries (Feinstone and Gust, 1997). The cause of increased occurrence of HAV in the waters tested in late

winter/early spring has been suggested to be a combination of factors including the viral stability in the environment, an increase in associated excretion of HAV from the surrounding communities into water sources and an increase in viral load after first run-off due to spring rains (Hollinger and Ticehurst, 1996; Taylor et al., 2001).

2.7 Bioaccumulation of HAV by shellfish

Shellfish, especially oysters, are filter feeders that can bioconcentrate human pathogens from contaminated growing waters (Wang et al., 2008). HAV could be retained in oysters for up to 1 month (Nappier et al., 2008). Enteric viruses including HAV were shown to accumulate in the gut of Pacific oysters harvested from New Zealand after 48 h of bioaccumulation, and poliovirus was mostly eliminated after a 23 h cleansing phase. On the contrary, HAV and norovirus were able to remain in the gut. In addition, bioaccumulation of poliovirus resulted in loss of infectivity, while HAV was still infectious (McLeod et al., 2009).

Investigations on target tissue for virus detection in oysters indicated that among different tissues, gills showed the highest positive rate (14.71%), followed by stomach (13.97%) and digestive diverticula (13.24%) in oyster samples from China (Wang et al., 2008). In mussels, HAV could be concentrated 100-fold in the filtration apparatus and hepatopancreas and persisted for 7 days. When feeding was eliminated, the uptake of HAV decreased to undetectable level, and the depuration of HAV also slowed down (Enriquez et al., 1992).

When grouped by month, both the outbreak and case data reveal two periods of increased illness: late spring and late fall. These incidents roughly coincide with times when bioaccumulation rates in shellfish are high. During certain times in spring (Burckhardt et al., 1992) and fall (Cabelli, 1988) in temperate waters, hard clams (*Mercenaria mercenaria*) accumulate viruses and other microbial indicators at a significantly higher rate than at other times of the year and thus can be

periodically contaminated with high levels of sewage-associated microorganisms, including microbial pathogens. This phenomenon is subsequently reflected in the human health effects data. The increased consumption of raw shellfish (particularly hard clams) during these periods may also be coincident with higher illness rates (Rippey, 1994).

2.8 Selection of HAV strain

HAV is unique among its family *Picornaviridae* with regard to its growth characteristics (Brack et al., 1998; Dotzauer et al., 1994; Gauss-Muller and Deinhardt. 1984; Jia et al., 1996; Vallbracht et al., 1984). The virus is hepatotropic *in vivo* and can infect a variety of primate and non-primate cell lines *in vitro*. In contrast to other picornaviruses, HAV exhibits a protracted replication cycle and normally establishes a persistent infection with low virus yields. Wild type HAV replicates slow in cell culture, and shows no cytopathic effect (CPE). Although more rapid replication and higher final virus titers are obtained with cell culture-adapted viruses, even these variants replicate considerably more slowly and less efficiently than other members of the *Picornaviridae* family. Replication of cell culture-adapted HAV is not detectable within the first few days after infection. The infection does not induce any visible CPE, and there is no evidence that HAV notably interferes with the macromolecular synthesis of its host cell.

However, since the 1980s, several cytopathogenic variants of HAV have been described (Anderson, 1987; Brack et al., 1998; Cromeans et al., 1987; Levine et al., 1993, Morace et al., 1993; Nasser and Metcalf, 1987; Venuti et al., 1985). These cytopathogenic variants are highly cell culture adapted and characterized by a rapid replication phenotype. Various studies indicated that in general the CPE and adaptation of HAV to growth in cell culture are associated with various mutations which are distributed over the 5' nontranslated region (5'NTR) and the P2 and P3

genomic regions and that therefore the CPE correlates with the overall efficiency of viral replication (Brack et al., 1998; Morace et al., 1993; Zhang et al., 1995).

Studies demonstrated that cytopathogenicity of HM175 strains correlates with high replication capacity, and suggested that the CPE is also caused by induced apoptosis (Brack et al., 1998; Gosert et al., 2000; Zhang et al., 1995). Most researchers use the cytopathic HAV strain HM175/18f (Bhattacharya et al., 2004; Blaise-Boisseau et al., 2010; Di Pasquale et al., 2010; Houde et al., 2007; Hu and Arsov, 2009; Love et al., 2008; Rigotto et al., 2009; Serracca et al., 2010) in their studies. HM175/18f is highly adapted to growth in cell culture with rapid replication cycle, requiring only 6-7 days for onset of CPE (Zhang et al., 1995).

FRhK-4 cell line (Bhattacharya et al., 2004; Blaise-Boisseau et al., 2010; Houde et al., 2007; Love et al., 2008; Millard et al., 1987; Rigotto et al., 2009; Villar et al., 2006) is frequently used for propagation of HAV. Most HAV strains show a strict host range for FRhK-4 cells and exhibit rapid growth and release of virus into cell culture supernatant (Graff et al., 1994; Tsarev et al., 1991). FRhK-4 cells are also more permissive than other cell lines for transfection of HAV genome (Emerson et al., 1992).

2.9 Cell culture and HAV propagation

FRhK-4 cells are usually grown in Eagle minimum essential medium or Dulbecco's modified Eagle's minimum essential medium with 5% fetal calf serum. Antibiotics such as gentamicin sulfate and kanamycin are usually supplemented for selective pressure, and HEPES and sodium bicarbonate are used for maintaining the pH. The FRhK-4 cells are usually washed three times with phosphate-buffered saline (PBS) and ~0.15 ml virus stock is allowed to absorb for 90 min to 3 h at 34°C~37°C. The virus inoculum is usually removed and replaced with maintenance medium with only 2% fetal calf serum. The cells are then maintained in a 5% CO_2

incubator at 34°C~37°C and kept until 75% of each monolayer is affected by virus cytopathology. The cultures are usually frozen at -20°C and thawed three times and centrifuged for 10~30 min at 1000 × *g* and the supernatant is stored at -20°C as virus stock (Dotzauer et al., 1994; Mbithi et al., 1990; Romalde et al., 1994).

2.10 HAV detection in food products

Although HAV foodborne disease is a significant problem, foods are seldom tested for viral contamination, and testing is usually limited to shellfish. Frequently, the cause of an outbreak is suspected to be of viral origin, but because of the lack of sensitive and reliable methods, this assumption can rarely be confirmed through direct isolation of the virus in the implicated foods (Calder et al., 2003; Sanchez et al., 2002). HAV detection in food is more difficult than for other viral agents because of the long incubation period of HAV infection. In addition, highly sensitive techniques are required to detect viruses in food samples, in which viral loads are typically much lower than those found in clinical samples (Costafreda et al., 2006). Other obstacles concerning routine virus detection in foods are the low efficiency of nucleic acid extraction procedures and the presence of inhibitors to the molecular reactions.

2.11 qPCR

Cell culture has not always been sensitive enough to detect low levels of HAV, when nucleic acid amplification techniques such as the PCR (polymerase chain reaction) offer a more sensitive approach to detection. qPCR (real-time polymerase chain reaction) was the most used method to detect viruses in water sample after concentration. It's fast, accurate and efficient. PCR is a method that allows exponential amplification of short DNA sequences (usually 100 to 600 bases) within a longer double stranded DNA molecule (Hunt, 2010). PCR entails the use of a pair of primers, each about 20 nucleotides in length, which are complementary to a defined sequence on

each of the two strands of the DNA. These primers are extended by a DNA polymerase so that a copy is made of the designated sequence. After making this copy, the same primers can be used again, not only to make another copy of the input DNA strand but also of the short copy made in the first round of synthesis (Hunt, 2010). This leads to exponential amplification (Fig 2.7).

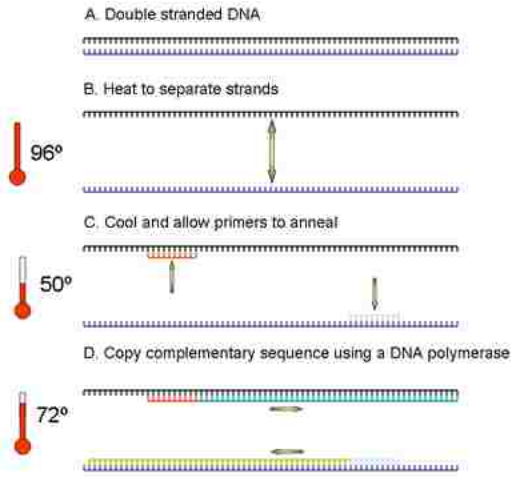

Fig. 2.7 Schematic diagram of PCR (Hunt, 2010)

In order to measure viral RNA, the method was extended using reverse transcriptase to convert RNA into complementary DNA (cDNA) which was then amplified by PCR (Fig 2.8).

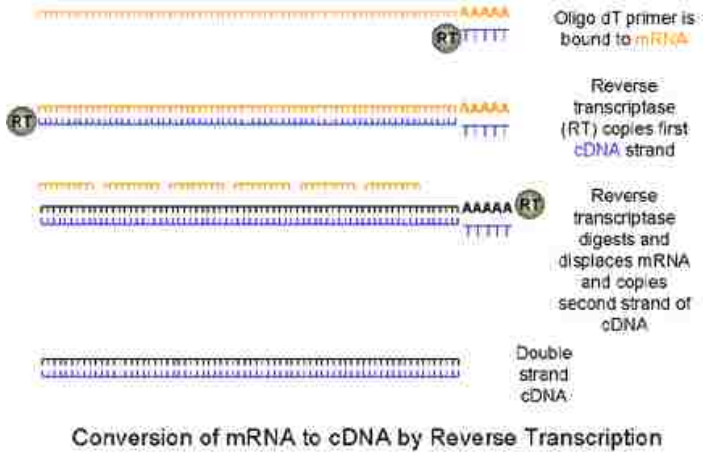

Fig. 2.8 Schematic diagram of reverse transcription (Hunt, 2010)

Quantitative PCR uses fluorescent dyes that give out fluorescence corresponding to the amplified DNA. The fluorescence intensity corresponds to the quantity of DNA in the sample.

Several different types of real-time PCR are currently available on the market, yet the most commonly used types are TaqMan® real-time PCR and SYBR® Green (Holland et al., 1991; Kutyavin et al., 2000; Pierce, 2003; Zipper et al., 2004). The SYBR® Green probe was the first to be used in real-time PCR. It binds to double-stranded DNA and emits light when excited (Zipper et al., 2004). TaqMan® real-time PCR relies on the 5'-3' exonuclease activity of Taq polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophore-based detection (Holland et al., 1991; Kutyavin et al., 2000; Pierce, 2003). TaqMan fluorescent probes consist of a fluorophore covalently attached to the 5'-end of the oligonucleotide probe and a quencher at the 3'-end (Fig 2.9).

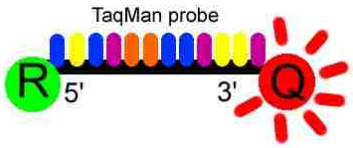

Fig. 2.9 TaqMan probe (Pierce, 2003)

The quencher molecule quenches the fluorescence emitted by the fluorophore when excited by the PCR cycler's light source via Fluorescence Resonance Energy Transfer. As long as the fluorophore and the quencher are in proximity, quenching inhibits any fluorescence signals. TaqMan probes are designed such that they anneal within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes the nascent strand, the 5' to 3' exonuclease activity of the polymerase degrades the probe that has annealed to the template (Holland et al., 1991; Kutyavin et al., 2000; Pierce, 2003). Degradation of the probe releases the fluorophore from it and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore (Fig 2.10).

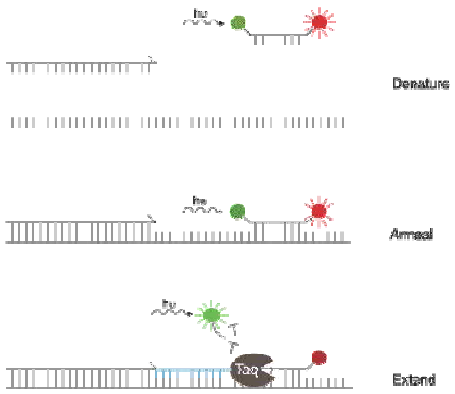

Fig. 2.10 Schematic diagram of TaqMan real-time PCR (Pierce, 2003)

Hence, fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template present in the PCR. The number of viral particles in an unknown sample can be obtained through the use of standard curve method (Hunt, 2010). A standard curve is constructed by amplifying a series of diluted viral RNA sample from a pure virus stock solution, in which the absolute quantity of virus is known (Fig 2.11).

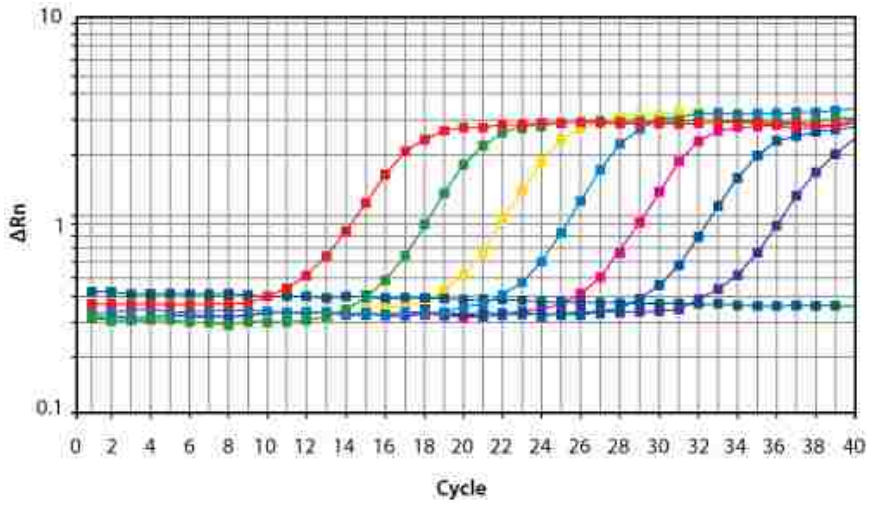

Fig. 2.11 PCR standard curve construction (www.sigmaaldrich.com)

The fluorescence intensity from each diluted RNA sample is then correlated with the absolute quantity of virus in that sample (Fig 2.12). By incorporating the fluorescence intensity of

an unknown sample to the standard curve, the absolute quantity of virus in the unknown sample could be calculated (Hunt, 2010).

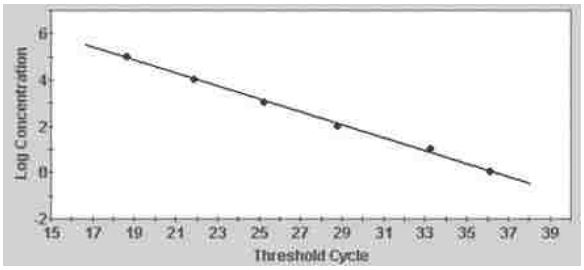

Fig. 2.12 Typical PCR standard curve (Li et al., 2006)

HAV contains a 7.5kb positive RNA composed of 5 functional regions within the nonenveloped virion. The most conserved region of HAV corresponds to the 5' noncoding region 5'NCR, being the regions spanning positions 66 to 95 and 227 to 262 (strain HM175) (Hollinger and Emerson, 2001). Although some HAV strains include some deletions in the region from position 99 to 207, it is otherwise quite well conserved between nucleotides 150 and 190 (Costafreda et al., 2006). Therefore 5'NCR is the most suitable region for design of primers (Costafreda et al., 2006; Costa-Mattioli et al., 2002b; Dubois et al., 2007; El Galil et al., 2004; Jothikumar et al., 2005; Sanchez et al., 2002). Some researchers also use the coding region for VP1 and VP3 protein for design of primers (Atmar et al., 1995; Costa-Mattioli et al., 2002a; Deng et al., 1994). The typical thermocycling condition of real-time PCR usually begins with reverse transcription at 45~50°C for 30 min to 1 h, followed by initial denaturation at 94~95°C for 3~5 min and then 35 to 50 cycles of amplification. The amplification cycle typically consists of denaturation at 94~95°C for 1 min, primer annealing at 49~60°C for 1 min, and extension at 60~72°C for 20 s to 1 min (Atmar et al., 1995; Costafreda et al., 2006; Costa-Mattioli et al., 2002a; Costa-Mattioli et al., 2002b; Deng et al., 1994; Dubois et al., 2007; El Galil et al., 2004;

Jothikumar et al., 2005; Le Guyader et al., 1994; Sanchez et al., 2002). The entire qPCR usually lasts for 2.5 h.

2.12 FRNA phage as indicator

HAV and other enteric viruses are the etiological agents most frequently associated with shellfish-associated infectious disease outbreaks that cause gastroenteritis. Although HAV can be elucidated by cell culture, the method is expensive and requires highly specialized equipment. Furthermore, certain cell lines will recover only certain virus type. Although molecular techniques for detection of HAV (Atmar et al., 1995; Lees et al., 1995) are available, these methods are usually expensive and time-consuming for routine screening.

Most countries impose legislative controls on the harvesting and placing on the market of live bivalve shellfish and use *Escherichia coli* as an indicator of fecal pollution in shellfish. While current legislation appears to be effective for controlling bacterial illness (West and Wood, 1985), viral infections associated with shellfish consumption continue to be reported (Chalmers and McMillan, 1995). Shellfish that are implicated in disease outbreaks with a viral etiology are frequently compliant with the *E. coli* standard (less than 230 *E. coli* per 100 g) (Chalmers and McMillan, 1995; Heller et al., 1986), particularly when the shellfish are purified prior to sale.

Indicator bacteria like fecal coliform are incapable of indicating the presence of enteric viruses (DePaola et al., 2007; Serracca et al., 2010). In contrast, male-specific (FRNA) bacteriophages have been proposed as indicators of enteric viruses. Clear correlation has been observed between FRNA phages and enteric viruses (Blaise-Boisseau et al., 2010; Doré et al., 2000). Unlike most human pathogenic enteric viruses that cannot be cultured, FRNA phages can be detected by culture and by molecular assays. Bacteriophage assay conditions are much simpler and cheaper than the enteric virus detection methods.

The F-specific RNA bacteriophages (FRNA bacteriophages) are a group of single-stranded RNA viruses with simple cubic capsids that are 24 to 27 nm in diameter (Furuse, 1987). FRNA bacteriophages have been classified into four groups (I, II, III and IV) on the basis of serological and physicochemical properties. These phages are are infectious to bacteria that possess the F-plasmid (Sobsey et al., 1995), and are relatively resistant to disinfectants, sunlight, heat treatment, and water and sewage-treatment processes (Havelaar and Hogeboom, 1984; Havelaar and Niewstad, 1985). The abundance of these phages in sewage, their genomic and physical resemblance of HAV, and the ease with which they can be enumerated make them attractive indicators of viral contamination in the environment (Havelaar et al., 1993). In addition, shellfish depuration has been shown to rapidly and effectively remove bacterial pollution indicators, but human enteric viruses are known to be more persistent.

While *E. coli* was absent, FRNA bacteriophages were often detected at high levels in oysters harvested at polluted sites. The frequency and degree of FRNA bacteriophage contamination were also closely associated with consumer health risk due to enteric viruses, as judged by the degree of harvest area pollution, the enteric virus content of shellfish, and the association with reported incidents of gastroenteric illness. Studies suggest that FRNA bacteriophages, unlike *E. coli*, are reliable and effective indicators of the possible presence of human enteric gastroenteritis viruses (Abad et al., 1997; Doré et al., 2000; Power and Collins, 1989; Schwab et al., 1998). Furthermore, unlike *E. coli*, the absence of FRNA bacteriophages appears to be a reliable indicator that enteric viruses are probably absent. Interestingly, FRNA bacteriophage contamination in depurated oysters also exhibited a seasonal trend that was consistent with the high-risk period for contamination by enteric viruses (Doré et al., 2000).

FRNA bacteriophages were also demonstrated to be much more resistant to chlorination and UV irradiation than bacterial indicators, and slightly more resistant than enteric viruses. The resistant nature of FRNA bacteriophage has been widely recognized (Hajenian and Butler, 1980; Harakeh and Butler, 1984; Havelaar and Nieuwstad, 1985; Tyrrell et al., 1995). Neither *E. coli* nor *Enterococci* are adequate indicators of virus removal during sewage disinfection. In contrast, FRNA bacteriophage was more resistant to inactivation than poliovirus under both laboratory and field conditions. The phage also showed similar behavior in both seeded and naturally contaminated samples. This suggests that FRNA bacteriophage would be a useful and conservative (fail-safe) model and indicator of virus inactivation during sewage chlorination and UV irradiation (Tree et al., 2003; Tree et al., 2005). They are more stable than human enteric viruses in environmental water (Sinton et al., 2002).

Since F-specific RNA phages exhibit high resistance to water purification process, they could be valuable models for viral inactivation by both UV and chemical disinfectants (Kott et al., 1974; Savichtchevaa and Okabe, 2006). Members of F-specific RNA phages are highly associated with fecal contamination from different hosts and domestic sewage (Rozen and Belkin, 2001; Savichtchevaa and Okabe, 2006; Scott et al., 2002). Therefore, the presence of F-specific phage in water was considered to be an index of sewage pollution (Osawa et al., 1981; Savichtchevaa and Okabe, 2006). FRNA bacteriophage was also associated with the levels of norovirus contamination (Lowther et al., 2008).

As a group I male-specific RNA coliphage that infects *Escherichia coli* (Nappier et al., 2008), MS2 is used widely as a surrogate for enteric viruses, and its presence has been proven to be a reliable indication of the presence of HAV. It can survive for a relatively long time in the environment, similar to that of HAV (Allwood et al., 2003; Huertas et al., 2003; Shin et al., 2003;

Tree et al., 2003; Katz and Margolin, 2007). MS2 is a member of the family *Leviviridae* and genus *Levivirus* and has a small, icosahedral capsid of 26.0 to 26.6 nm in diameter (Van Duin, 1988) (Fig 2.13). It infects *E. coli* by attaching to the sex pili of *E. coli* and multiplies inside the bacteria by making use of some or all of the host biosynthetic machinery. Its 3.5 kb single stranded RNA genome acts as messenger RNA and is responsible for encoding lysis protein that lyses the host bacteria upon completion of assembly (Carter et al., 2007).

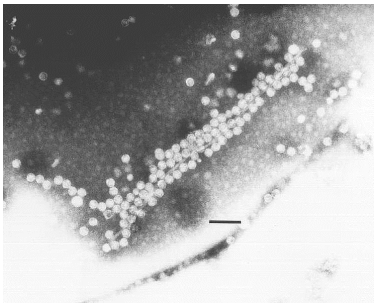

Fig. 2.13 Electron microscopy of bacteriophage MS2 (Ackermann, 1996).

2.13 Virus concentration methods

Numbers of viruses and phages in contaminated food or water are usually too low for detection. Unfortunately, even one virion is sufficient to cause illness (Cuthbert, 2001). Therefore, large volumes of water (10-20 L) need to be concentrated before analysis can be carried out. Different types of filters and filtration methods have been used to collect and concentrate viral particles from water samples (Fong and Lipp, 2005; Gantzer et al., 1999; Griffin et al., 2003; Lipp et al., 2001; Pallin et al., 1997). Because of the small size of viral particles, mechanical filtration is often not possible; therefore, adsorption-elution methods are employed. These involve manipulation of charges on the virus surface, using pH changes to maximize their adsorption to charged filters (Fong and Lipp, 2005; Lipp et al., 2001; Pallin et al., 1997).

Adsorption-elution of viruses with an electropositive filter is one of the most commonly used techniques (American Public Health Association, 1995; Fong and Lipp, 2005; Katayama et

al., 2002). This method has been applied to tap water, ground water, river water, lake water, secondarily treated sewage, and marine water (Abbaszadegan et al., 1993; Logan et al., 1980; Logan, et al., 1981; Nupen and Bateman, 1985; Reynolds et al., 1993; Shri and Gerba, 1983; Sobsey et al., 1985). These filters require no manipulation of pH because most enteric viruses are negatively charged at ambient pH (Lipp et al., 2001). However, electropositive filters are easily clogged and have low recovery rates for viruses in marine water; the presence of salt and alkalinity of seawater cause low absorption of viruses to the filters (Lukasik et al., 2000).

Katayama and others (2002) developed a new series of procedures to concentrate viruses by adsorption to and elution from a negatively charged membrane filter, with the insertion of an acid rinse step for removing cations and other inhibitors without eluting the viruses from the membrane between the adsorption and elution steps. In this method, as much as 2 L of seawater was filtered by electronegative membrane filter (90 mm diameter, 0.45 µm pore size), followed by an acid rinse step using 500 ml of hydrosulfuric acid solution (0.5 mM) and a base elution step using 10 ml of sodium hydroxide solution (1 mM). The method proceeded with additional acid neutralization and a secondary concentration step to reduce the sample volume to 2 ml. In the acid rinse procedure at pH 3.0, in which viruses become positively charged since they normally have a higher isoelectric point, viruses are expected to attach themselves directly to the filter, while the cations are eluted from it. The acid rinse step was shown to increase elution and detection efficiency possibly due to reduction of inhibitors in the recovered eluates from original environmental water samples. In the subsequent alkali elution, the charge of virus surfaces was converted from positive to negative, which allowed viruses to be eluted from the negatively charged filter (Katayama et al., 2002).

Under ambient conditions, most of the enteric viruses are negatively charged and are known to adsorb to an electronegative filter in the presence of Mg^{2+} or other multivalent cations, or under acid conditions, while the recovery of viruses is not always easy (Fong and Lipp, 2005; Sobsey et al., 1973; Sobsey, 1995). Electronegative filters show higher virus recoveries from marine water and waters of high turbidity than do electropositive filters (Enriquez and Gerba, 1995; Fong and Lipp, 2005; Katayama et al., 2002; Lipp et al., 2001, Lukasik et al., 2000). The use of an electronegative has subsequently became a popular method in virus concentration from seawater (Brooks et al., 2005; Gersberg et al., 2006; Villar et al., 2006). Villar and others (2006) modified this method by adding $MgCl_2 \cdot 6H_2O$ (1200 mg/l in 500 ml sample) and adjusting the pH to 5.0. The samples were filtered through negatively charged filters with a pore size of 0.45µm and a urea-arginine phosphate buffer (pH 9.0) was used for viral elution. The eluates were flocculated with additional $MgCl_2 \cdot 6H_2O$ and precipitated by centrifugation for 30 min at 3000 ×g, and the pellets were solubilized in 800 µl of distilled sterile water.

Ultrafiltration methods such as vortex flow filtration (VFF) and tangential flow filtration (TFF) are alternatives to adsorption-elution techniques and have been shown to be efficient in recovering viruses from marine water (Fong and Lipp, 2005; Griffin et al., 2003; Paul et al., 1991). Both filtration devices utilize a flow pattern that forces water through a cylindrical filter with pressure while keeping and retaining particles from filters to avoid clogging (Fong and Lipp, 2005; Paul et al., 1991). These methods require minimal manipulation of water; samples can be processed under natural pH, and an elution step is not needed (Fong and Lipp, 2005; Jiang et al., 2001). The typical volume of water processed is 20 L, which is concentrated to ~50 ml (Fong and Lipp, 2005; Griffin et al., 2003). TFF requires pre-filtration of water samples to remove plankton and suspended solids. VFF has been shown to be more time-efficient because pre-filtration of

samples is not required, and it has a higher viral recovery rate than TFF, but it tends to concentrate more PCR inhibitors with the viruses (Fong and Lipp, 2005; Jiang et al., 2001). However, both VFF and TFF are more costly and time-consuming than adsorption-elution because of the high cost of equipment and limitations on the volume of sample that can be concentrated at one time.

Concentrated or eluted water samples usually are further concentrated and purified to reduce the final volume of samples to 1 or 2 ml for processing (Fong and Lipp, 2005; Haramoto et al., 2004; Jiang et al., 2001; Katayama et al., 2002; Lipp et al., 2001; Noble and Fuhrman, 2001). Commonly used secondary concentration methods include organic flocculation, PEG precipitation, and centrifugal ultrafiltration (Fong and Lipp, 2005; Jiang et al., 2001; Katayama et al., 2002; Lipp et al., 2001). In various concentrating methods, beef extract was often used as an eluate from various adsorbents (Logan et al., 1980; Payment and Trudel, 1985; Stetler et al., 1992; Yano et al., 1993). However, contents of beef extract are suspected to have some inhibitory effect on PCR detection for viruses, especially after reconcentration. In organic flocculation, buffered beef extract is used to precipitate viruses from concentrated samples by reducing the pH to 3.5. The precipitate is then centrifuged to form a pellet before being dissolved in sodium phosphate. The PEG precipitation procedure consists of precipitating viral particles by addition of 0.5 M NaCl and 7% PEG to beef extract with constant stirring for 2 h at 4°C followed by centrifugation at 10000~12000 × *g* for 30 min. The virus pellet is then resuspended in Tris-buffered saline (Enriquez and Gerba, 1995; Guévremont et al., 2006; Zhou et al., 1991). Again, the use of beef extract in these procedures has been reported to cause inhibitory effects in PCR assays (Arnal et al., 1999; Fong and Lipp, 2005).

We have developed a virus concentration method that does not require a secondary concentration step or the need to modify the pH or salinity of the seawater, and could be used for rapid virus detection in seawater.

2.14 References

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Chapter 3. An improved plaque assay of bacteriophage MS2

3.1 Introduction

Bacteriophage MS2 is a group I male-specific RNA coliphage that infects *Escherichia coli* (Nappier et al., 2008). Because its structure is similar to that of many pathogenic enteric viruses, and is as resistant to conditions in natural water environments and water treatment processes, MS2 is used widely as a surrogate for enteric viruses, and its survival characteristics under various disinfection treatments have been evaluated by extensive research (Allwood et al., 2003; Bae and Schwab, 2006; Cho et al., 2005; Mamanea et al., 2007; Shin and Sobsey, 2003; Sjogren and Sierka, 1994; Templeton et al., 2006; You et al., 2003). MS2 is a member of the family *Leviviridae* and genus *Levivirus* and has a small, icosahedral capsid of 26.0 to 26.6 nm in diameter (Van Duin, 1988). It infects *E. coli* by attaching to the sex pili of *E. coli* and multiplies inside the bacteria by making use of some or all of the host biosynthetic machinery. Its 3.5 kb single stranded RNA genome acts as messenger RNA and is responsible for encoding lysis protein that lyses the host bacteria upon completion of assembly (Carter et al., 2007).

MS2 is usually quantified by counting infectious units via plaque assay (Cho et al., 2005; Dawson et al., 2005; Mamanea et al., 2007; O'Connell et al., 2006; Shin and Sobsey, 2003; Sjogren and Sierka, 1994; Templeton et al., 2006; You et al., 2003). Plaque assay applies the ability of bacteriophages to kill the host bacteria and allow phages to propagate in a confluent lawn of bacterial host cells immobilized in a layer of agar. After incubation, a circular transparent area of lysed cells will develop, and each plaque represents a single phage particle in the original sample. The bacteriophages are usually mixed with host bacteria in molten agar and poured on top of a bottom agar layer. Bottom agar usually contains 1%-1.5% agar while top agar contains 0.45%-0.75% agar (Cho et al., 2005; Dawson et al., 2005; Mamanea et al., 2007; O'Connell et al.,

2006; Shin and Sobsey, 2003; Sjogren and Sierka, 1994; Templeton et al., 2006; Thompson and Yates, 1999; You et al., 2003). Alternatively, bacteriophages are spotted on top of the agar (American Type Culture Collection, 2010; Lillehaug, 1997; Nappier et al., 2006).

The plaque assay is used commonly for detection of MS2 in treated drinking water, waste water, and marine water as an assessment of the water quality (Cho et al., 2005; Dawson et al., 2005; Grabow and Coubrough, 1986; Mamanea et al., 2007; O'Connell et al., 2006; Shin and Sobsey, 2003; Sjogren and Sierka, 1994; Templeton et al., 2006; Thompson and Yates, 1999; You et al., 2003). The plaque assay can also be used to establish the absolute quantity of MS2 during the construction of a PCR standard curve (Dreier et al., 2005; Furiga et al., 2011; Gentilomi et al., 2008). Current plaque assay methods provide a simple and direct approach in MS2 enumeration and work well within the need of water quality assessment. However, during the attempted construction of a PCR standard curve, we discovered that existing plaque assays often produce small and hazy plaques; thus resulting in variation of plaque count and increased variation between replicates. Because the accurate quantification is of MS2 is extremely critical to the establishment of a standard curve, we found it necessary to optimize the current plaque assay for better visibility and lower variation. In an attempt to improve the visibility and precision of the current plaque assay, spread plate technique was introduced, which was commonly used in bacterial enumeration, as contrary to the pour plate technique commonly used in existing plaque assay methods. Other parameters that influence the outcome of the plaque assay were also compared. This report summarizes a study of factors that have important effects on the formation of plaques, and describes an optimized plaque assay that produces large and clear plaques with good consistency.

3.2 Materials and methods

3.2.1 Bacterial and viral strains and growth media

E. coli (ATCC 15597) was grown at 37°C for 6 h. The growth media of *E. coli* contained 10 g tryptone, 1 g yeast extract, 8 g NaCl per liter of medium. After the medium was cooled to 50°C, the following supplements glucose (0.1%), $CaCl_2$ (2 mmol ml^{-1}) and thiamine (10 µg ml^{-1}) were added to the cooled medium. Bacteriophage MS2 (ATCC 15597-B1) was propagated in the exponentially growing *E. coli* culture overnight (16 h) at 37°C.

3.2.2 Purification of bacteriophage

An overnight culture (16 h) of MS2 was centrifuged at 2320× *g* for 10 min to separate the host cell debris and the bacteriophage. The supernatant was filtered through a 0.45 µm filter and used as a stock solution. Serial dilutions were made and all dilutions as well as the stock were stored at 4°C.

3.2.3 Single layer plaque assay

The plaque assay medium contained 10 g tryptone, 1 g yeast extract, 8 g NaCl per liter of medium with the addition of agar. Two agar concentrations, 0.6% and 1.2% were tested for adjustment of agar softness. The agar was boiled and kept at ~50°C before *E. coli* 6 h culture was seeded at a ratio of 1:100. The seeded agar was separated into two portions; one portion for pour plate and the other portion for spread plate. The portion for pour plate was inoculated with MS2 dilution of 10^3 plaque forming units per milliliter (PFU ml^{-1}) to a 0.3% final concentration, before the agar was poured onto 100×15 mm Petri dishes, at volumes 10 ml, 20 ml and 30 ml, for adjustment of agar thickness. To prevent condensation on the plates, the hardened agar was dried under the biological safety cabinet, with the sterile air flowing directly above the agar. The portion for spread plate was poured onto 100×15 mm Petri dishes, at volumes 10 ml, 20 ml and 30 ml,

before 30 µl MS2 dilution (10^3 PFU ml^{-1}) was spread on top with a hockey stick (bent glass rod). The same conditions were tested with supplementation of 0.1% glucose, 2 mmol ml^{-1} $CaCl_2$ and thiamine (10 µg ml^{-1}). All the plates were incubated at 37°C overnight (16 h) before examined for plaque formations.

3.2.4 Double layer plaque assay

The double layer plaque assay medium contained the same ingredients as the single layer assay. The plaque assay plates consisted of two layers of agar; a bottom agar with 1% or 1.2% agar and a top agar with 0.45% or 0.6% agar. The bottom agar was boiled and cooled before poured into 100×15 mm petri dishes at volumes 10 ml, 20 ml and 30 ml, for adjustment of agar thickness, and dried under biological safety cabinet. The top agar was boiled and kept at ~50°C before *E .coli* 6 h culture was seeded at a ratio of 1:100. The seeded agar was separated into two portions; one portion for pour plate and the other portion for spread plate. The portion for pour plate was inoculated with MS2 dilution (10^3 PFU ml^{-1}) to a 0.3% final concentration, before the agar was poured on top of the bottom agar. Plates with 10 ml and 20 ml bottom agar received 10 ml top agar; while plates with 30 ml bottom agar received 10 ml, 15 ml and 20 ml top agar. The double layer agar was then dried under biological safety cabinet again. The portion for spread plate was poured on top of the bottom agar, before 30 µl MS2 suspension (10^3 PFU ml^{-1}) was spread on top with a hockey stick. The same conditions were tested with supplementation of 0.1% glucose, 2 mmol ml^{-1} $CaCl_2$ and thiamine (10 µg ml^{-1}). All the plates were incubated at 37°C overnight (16 h) before examined for plaque formations.

3.2.5 Comparison of different plaque assay conditions

All the plates were examined for plaque formations after overnight incubation (16 h) and categorized by the differences in agar composition, supplementation, and position of MS2.

Comparisons were carried out based on the size, clarity and number of plaques on each plate. The optimal plaque assay was selected for maximum plaque size, clarity, and plaque count.

3.2.6 Comparison of optimized plaque assay with existing plaque assay methods

The optimized plaque assay was compared with existing plaque assay methods from American Type Culture Collection (2010), Cornax (1990), Lillehaug (1997) and O'Connell (2006) (Table 3.1). Most plaque assay methods use pour plate technique and mix the bacteriophage with its host bacteria inside the agar (Cho et al., 2005; Dawson et al., 2005; Dore´ et al., 2003; Mamanea et al., 2007; O'Connell et al., 2006; Shin and Sobsey, 2003; Sjogren and Sierka, 1994; Templeton et al., 2006; Thompson and Yates, 1999; You et al., 2003). The methods above were chosen because they offered more detailed descriptions of the plaque assay than other publications. Ten plates were prepared in each plaque assay method and the same *E. coli* culture and MS2 dilution were used in all the plates. Plates displaying 10 to 100 plaques were used for enumeration and the mean and standard deviation of plaque count were calculated. Two plaques were selected from each plate at random, and the plaque sizes were measured with a ruler. Plaque assay plates were prepared according to instructions from each method. However, while American Type Culture Collection (2010) and O'Connell (2006) used bacteriophage MS2 as target organism, Cornax (1990) and Lillehaug (1997) focused on other types of bacteriophages, but the agar compositions used in their methods were similar to less detailed methods using MS2 as model organism (Dawson et al., 2005; Doré et al., 2003; Hot et al., 2003; Mamanea et al., 2007; Shin and Sobsey, 2003; Sjogren and Sierka, 1994; Templeton et al., 2006; Thompson and Yates, 1999; You et al., 2003). In addition, none of the existing methods specified the detailed composition of the agar used in the assay: American Type Culture Collection, Cornax and O'Connell did not specify the volume of bottom agar; Lillehaug and O'Connell did not specify the volume of bacteriophage suspension;

O'Connell did not specify the volumes of top agar and host bacteria. In order to compensate for the vagueness, 0.1% glucose, 2 mmol ml^{-1} $CaCl_2$ and 10 µg ml^{-1} thiamine; 20 ml bottom agar, 10 ml top agar and 30 µl bacteriophage suspensions were used in every plate unless instructed otherwise. Moreover, American Type Culture Collection included a double layer method and a single layer method, while Lillehaug included a "spotting" method and a "pouring" method. Since the double layer method of American Type Culture Collection and the "spotting" method of Lillehaug were rather similar, only the Lillehaug method was performed.

Table 3.1 Agar compositions of plaque assay from different existing methods.

Reference	Bottom agar	Top agar	*E. coli* culture: top agar	MS2 suspension	MS2 suspension method
Optimized plaque assay	10 ml 1% agar	10 ml 0.45% agar	1:100	30 µl	Spread plate
American Type Culture Collection	20 ml 1.5% agar	none	1 ml/petri dish	100 µl	Spotting
Cornax	20 ml 1.4% agar	3 ml 0.5% agar	1:3	30 µl	Pour plate
Lillehaug (pour)	30 ml 1% agar	2.5 ml 0.3% agar	1:250	30 µl	Pour plate
Lillehaug (spot)	30 ml 1% agar	2.5 ml 0.3% agar	1:250	30 µl	Spotting
O'Connell	20 ml 1.5% agar	10 ml 0.5% agar	1:50	30 µl	Pour plate

3.2.7 Statistical analysis

Plaques were enumerated from all ten plates of each method, and the plaque count of the optimized plaque assay was compared to that of the other methods using ANOVA with JMP software.

3.3 Results

3.3.1 Spread plate vs. pour plate

The effect of using spread plate technique was significant, resulting in increase of plaque size by approximately 50% (Table 2). Regardless of agar compositions, plates with MS2 spread on top always had clearer plaques than plates with MS2 mixed in (Fig 1A vs. Fig 1B). While the effect of spread plate technique wasn't profound in plates with 1.2% bottom agar since the agar composition was less than optimal, the increase of plaque size and plaque count were more intense in plates with 1% bottom agar; spread plates had 4.9 ± 2.1 times more plaques than pour plates, and the plaques were approximately 0.5 mm larger. It is possible that in pour plates the bacteriophages were trapped in the agar and thus were unable to infect the host bacteria. In addition, the lyse zones inside the agar may be less visible than lyse zones on top of the agar.

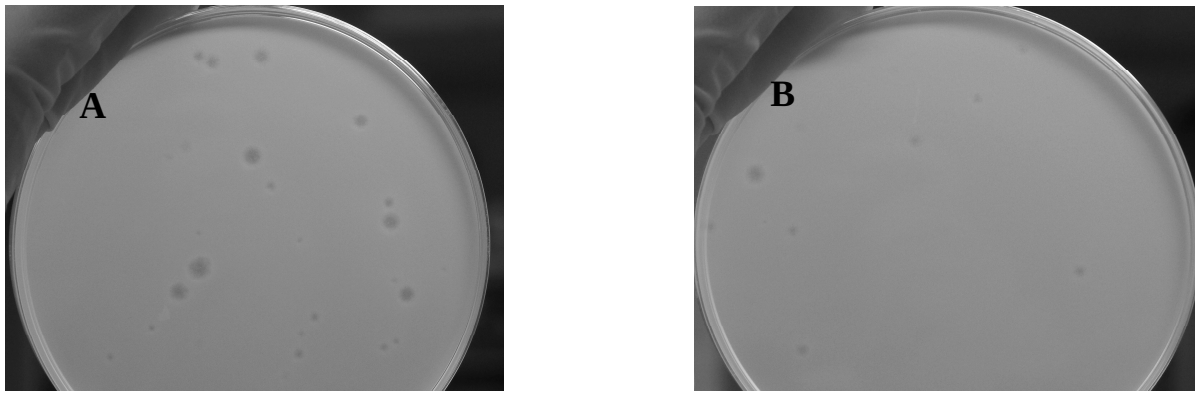

Fig. 3.1 Spread plate vs. pour plate. (A) 30 ml supplemented 1% bottom agar overlaid with 10 ml supplemented 0.45% agar with 30 µl MS2 spread on top; (B) same composition of agar as in A, but with 30 µl MS2 mixed inside top agar.

3.3.2 Addition of supplements

Addition of supplements to the agar also resulted in increased plaque visibility. On average plates with supplements produced 5 times more plaques than plates without supplements. Although the plaque size did not have a substantial increase, the visibility of the plaques was enhanced

significantly (Fig 2A vs. Fig 2B). Supplement addition followed the exact concentrations recommended by American Type Culture Collection (2010).

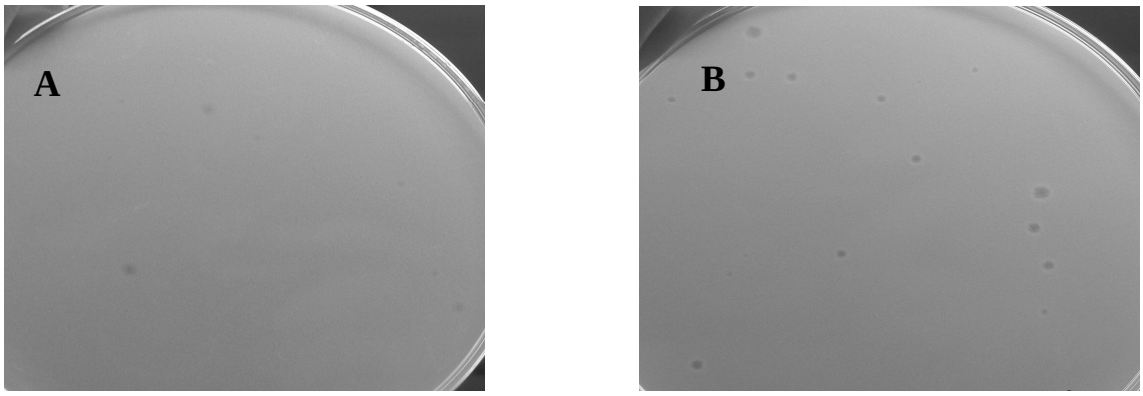

Fig. 3.2 Supplement addition. (A) 20 ml un-supplemented 0.6% agar with 50 µl MS2 spread on top; (B) same composition of agar as in A, but with supplements.

3.3.3 Softness of agar

The softness of the agar affected the visibility and size of the plaques to a great extent. Plates with a single layer of 1.2% agar often failed to produce any plaques, while plates with a single layer of 0.6% agar displayed up to 15 plaques. When a double layer agar was used, the combination of soft agar layers also boosted the plaque size and count. Plates with 1% bottom agar and 0.45% top agar often displayed more than 30 plaques, and the plaques were the clearest among all conditions; while plates with 1.2% bottom agar and 0.6% top agar often displayed less than 10 plaques which were vague and small (Fig 3A vs. Fig 3B). On average, plates with 1% bottom agar and 0.45% top agar displayed 10.9 ± 8.1 times more plaques than plates with 1.2% bottom agar and 0.6% top agar, and the plaques were approximately 0.6 mm larger. Using softer agar for both layers provided a higher diffusion rate for the phages, thus increasing plaque visibility.

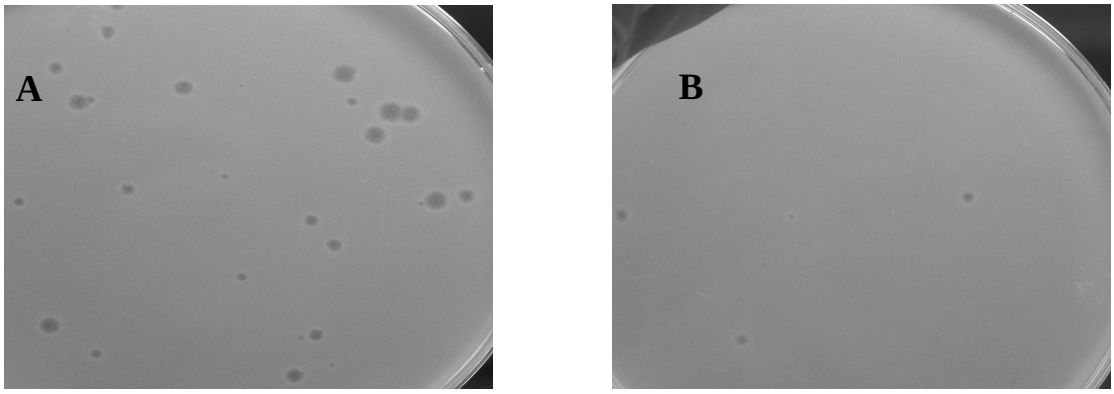

Fig. 3.3 Soft agar vs. hard agar. (A) 10 ml supplemented 1% agar overlaid with 10 ml supplemented 0.45% agar with 30 µl MS2 spread on top; (B) 10 ml supplemented 1.2% agar overlaid with 10 ml supplemented 0.6% agar with 30 µl MS2 spread on top.

3.3.4 Thickness of agar

The thicknesses of both bottom and top agar had a great impact on the visibility of plaques. The effect of agar thickness was more profound in plates with 1% bottom agar and 0.45% top agar, since plates with 1.2% bottom agar and 0.6% top agar hardly produced any visible plaques. Plates with thin agar layers (10 ml agar per plate) had approximately 1.4 times (11 in count) more plaques than plates with thick agar layers (20-30 ml agar per plate). Although the size did not change much, plaques produced by thick agar plates were far less clear than plaques from thin agar plates (Fig 4A vs. Fig 4B); in addition, when the top agar was increased to 15 ml per plate, multiple bubbles were produced inside the top agar, making the plate count confusing (Fig 4C). Decreasing the thickness of both layers increased the contrast between infected host cells and uninfected cells and improved the visibility of the plaques.

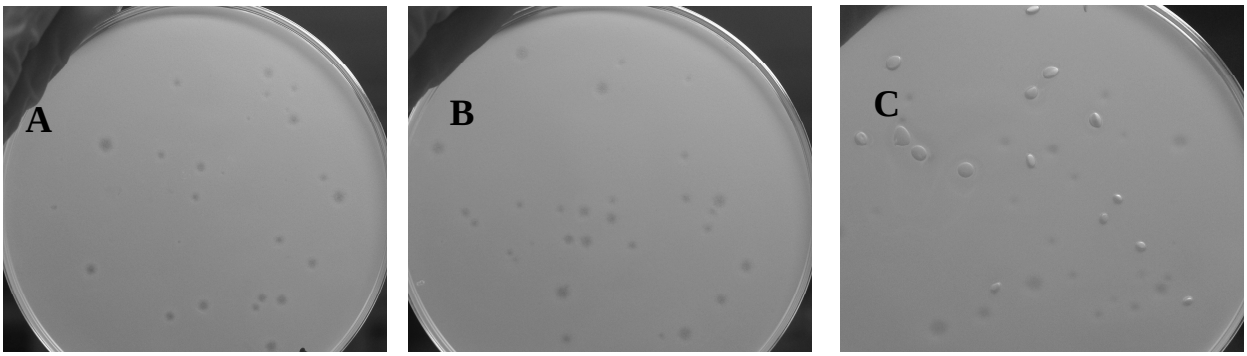

Fig. 3.4 Thin agar vs. thick agar. (A) 20 ml supplemented 1% agar overlaid with 10 ml supplemented 0.45% agar with 30 µl MS2 spread on top; (B) 30 ml supplemented 1% agar overlaid with 10 ml supplemented 0.45% agar with 30 µl MS2 spread on top; (C) 30 ml supplemented 1% agar overlaid with 20 ml supplemented 0.45% agar with 30 µl MS2 spread on top.

3.3.5 The optimized plaque assay

Among all the conditions tested, the optimized plaque assay was a supplemented thin bottom agar (10 ml 1% agar in 100×15 mm plate) with a supplemented thin top agar (10 ml 0.45% agar in 100×15 mm plate) with 30 µl MS2 suspension spread on top. The optimized plaque assay displayed the largest (2 mm) and clearest plaques when compared to other conditions (Table 3.2).

3.3.6 Comparison with existing plaque assays

The optimized plaques assay was compared to 4 different existing method plaque assays (Cornax et al. 1990; Lillehaug. 1997; O'Connell et al. 2006; American Type Culture Collection. 2010). All the plates were examined for plaques after 16 h of incubation at 37°C. Repeated experimentation showed that plaque formation remained stable after 16 h, and increasing the incubation time up to 48 h did not alter the size or appearance of any plaques. The optimized plaque assay had a medium plaque size, while the pouring method from Lillehaug (1997) had the largest plaque size and the method from American Type Culture Collection (2010) had the smallest plaque size (Table 3.3).

Table 3.2 Comparison of plaque size of different plaque assay conditions.

Bottom agar	Top agar	Supplements	MS2 suspension method	Mean plaque size (mm)
10 ml 1% agar	10 ml 0.45% agar	Yes	Spread plate	1.5
30 ml 1% agar	10 ml 0.45% agar	Yes	Spread plate	1.2
10 ml 1.2% agar	10 ml 0.6% agar	Yes	Spread plate	0.8
30 ml 1.2% agar	10 ml 0.6% agar	Yes	Spread plate	0.8
10 ml 1% agar	10 ml 0.45% agar	Yes	Pour plate	1
10 ml 1.2% agar	10 ml 0.6% agar	Yes	Pour plate	1
30 ml 1.2% agar	10 ml 0.6% agar	Yes	Pour plate	No plaques
10 ml 1.2% agar	10 ml 0.6% agar	No	Pour plate	1
30 ml 1% agar	10 ml 0.45% agar	No	Pour plate	0.8
30 ml 1.2% agar	10 ml 0.6% agar	No	Spread plate	0.8
10 ml 1.2% agar	10 ml 0.6% agar	No	Spread plate	1
10 ml 1% agar	10 ml 0.45% agar	No	Spread plate	0.8
30 ml 1.2% agar	10 ml 0.6% agar	No	Spread plate	No plaques

Table 3.3 Plaque size comparison of different plaque assay methods.

Reference	Replicates	Plaque size (mm)
Optimized plaque assay	20	2.0 ± 0.83
American Type Culture Collection	20	0.7 ± 0.19
Cornax	20	1.2 ± 0.57
Lillehaug (pour)	20	3.3 ± 1.39
Lillehaug (spot)	20	2.8 ± 1.18
O'Connell	20	1.6 ± 0.66

Plates from the optimized plaque assay had the highest clarity and it was extremely easy to distinguish individual plaque; plates from American Type Culture Collection method had only a very thin lawn of host bacteria, and the plates were almost transparent, thus counting the plaques

was quite challenging; plates from both methods of Lillehaug produced large plaques, but plaques were often clumped together (Fig 3.5).

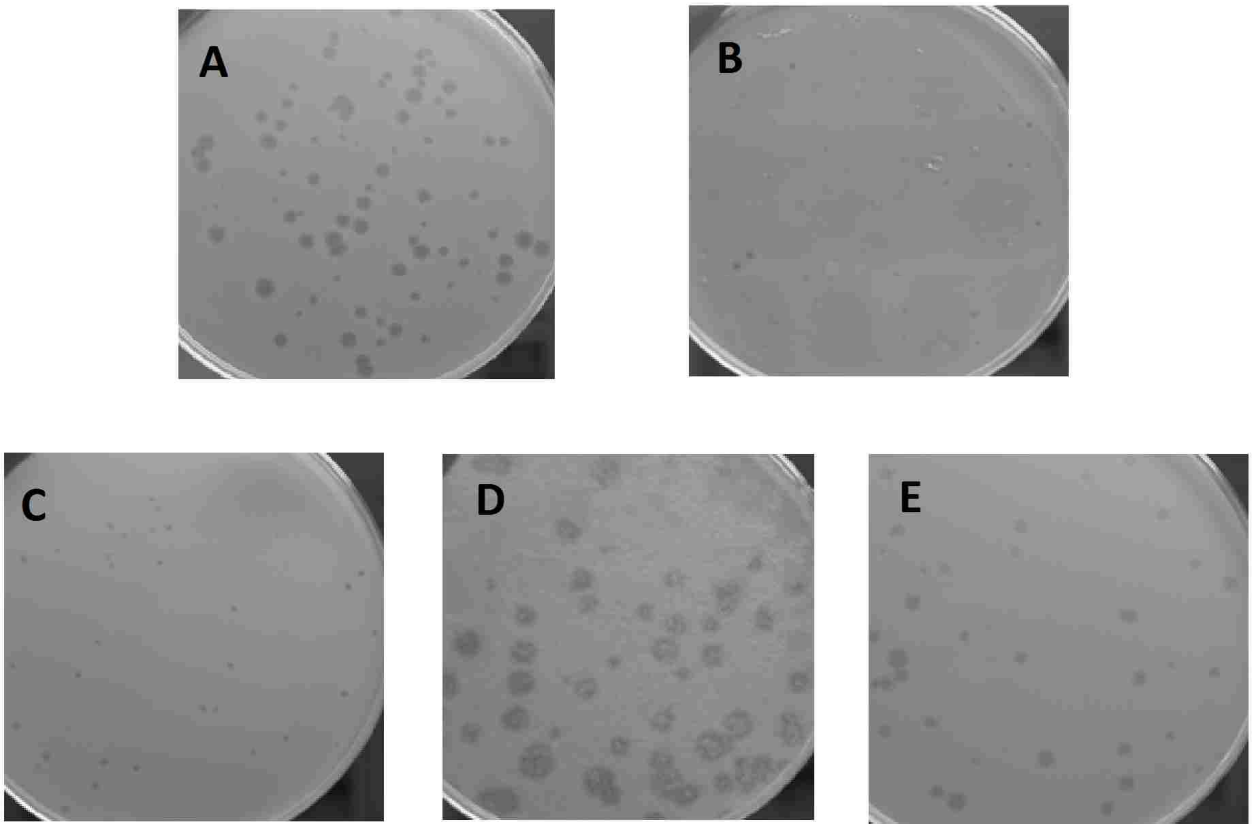

Fig. 3.5 Comparison of optimized plaque assay with existing plaque assay methods. (A) the optimized plaque assay; (B) method of American Type Culture Collection; (C) "pouring" method of Lillehaug; (D) method of Cornax; (E) method of O'Connell.

Despite the clarity of plaques in Lillehaug's method, each plaque may have generated from multiple bacteriophages, which could explain the large size and low count of the plaques. In addition, spotting phage suspension on top of a soft agar was quite difficult to handle, and may have led to uneven distributed phages. Since the same MS2 dilution was used in all the methods, theoretically the plaque count obtained from each method should be the same. The significant differences in plaque count between methods thus stem from the differences in accuracy and consistency of each method. The plaque count from the optimized plaque assay had the highest

PFU per plate among all methods, and it was significantly different ($P<0.01$) from all the other methods. Moreover, the plaque count from the optimized plaque assay had the smallest coefficient of variation, indicating highest precision. Even though Cornax's method produced an overall high plaque count, its coefficient of variation was second to largest, indicating a poor consistency over repeated trials (Table 3.4).

Table 3.4 Statistical analysis of plaque count among different plaque assay methods.

Reference	Replicates [a]	PFU per plate	Coefficient of variation	Prob>F [b]
Optimized plaque assay	10	81.9 ± 7.2	8.8%	1
American Type Culture Collection	9	22.9 ± 12.1	52.8%	<0.0001
Cornax	10	66.9 ± 28.7	42.9%	N/A [c]
Lillehaug (pour)	9	62.8 ± 5.5	8.8%	<0.0001
Lillehaug (spot)	10	43.4 ± 10.4	24.0%	<0.0001
O'Connell	10	67.3 ± 14.5	21.5 %	0.0104

[a] One replicate of ATCC's method and one replicate of Lillehaug D.'s "pouring" method were discarded due to the poor visibility of the plates.
[b] The PFU per plate of each method was compared to the optimized plaque assay using ANOVA (analysis of variance) and a Prob>F value of less than 0.05 indicates significant difference between that method and the optimized plaque assay.
[c] The method of Cornax was not compared to the optimized plaque assay because its standard deviation was too large for the statistics to be reliable.

3.4 Discussion

The reliability and accuracy of plaque assay technique is highly dependent on the consistency of the plaque count, which is in turn affected by the visibility of the plaques. The composition of the double layer agar directly influences plaque formation. In a less than optimal condition, the lyse zones form plaques are tiny and hazy, possibly because the bacteriophages were unable to infect and lyse the host cells (Fig 3.5B, 3.5D and 3.5E). The poor visibility of the plaques would result in underestimation of the phage quantity, since some plaques may be too tiny or vague to be seen. While the enumeration of phages in an environmental sample focusses more on

the presence/absence of phages and existing plaque assay methods work well, quantification of bacteriophages calls for more precise methods. The optimized plaque assay increased the plaque size and clarity greatly and made it easier to distinguish plaques from background.

Lillehaug (1997) suggested that a softer and thinner top layer of agar stimulates higher rate of phage diffusion. Since a plaque extends throughout three dimensions, and the sphere of infected and lysed cells in a thick layer of top agar thus may be easily camouflaged by uninfected host cells in the plaque–lawn boundary region, a thin top agar provides better visual contrast. The bottom agar, which provides nutrients, buffers against reduction in pH and helps to maintain a soft and moist lawn, also provides better visibility when it's soft and thin. In this study, the thickness of the agar was controlled by the volume of molten agar placed in the Petri dish. The combination of a thin bottom agar with a thin top agar demonstrated a significant improvement on the plaque visibility and counts.

Position of the bacteriophage MS2 determines if the lysis of the host cells would be visible as a plaque. When MS2 is contained inside the agar, it could be trapped in the agar and not able to infect the *E. coli*. American Type Culture Collection (2010) suggested that mixing the MS2 suspension with the molten agar would result in small and hazy plaques. American Type Culture Collection (2010) and Lillehaug (1997) both offered a solution to the problem by spotting the phage suspension on top of the agar. However, the homogeneity of the phage distribution was very difficult to achieve with the spotting technique. As a result, the total plaque count was lower compared to other methods. An alternative solution offered in this study showed that spreading the phage suspension on top of the agar could solve the homogeneity issue. Since a soft thin agar was used, the hockey stick needed to be applied onto the surface gently which would avoid breaking the agar. Theoretically, each plaque represents a single phage particle in the original sample.

However, based on observations from the plaque assays, phages sometimes aggregate, as evidenced by formation of clumps (Fig 3.5C), and variation of size within the same plate (Fig 3.5A and 3.5E).

Supplements used in this study (glucose, $CaCl_2$ and thiamine) have significant impacts on the outcome of the plaque assay due to their roles in bacteria metabolism. Glucose is used by *E. coli* as a carbon source; it not only provides energy but also serves as a building block. Glucose can be used for synthesis of amino acids, vitamins, nucleotides, and cell wall constituents. Thiamine is the cofactor of transketolase, an important enzyme in the pentose phosphate pathway utilized by *E. coli* for glucose catabolism. Pentose phosphate pathway enables the bacteria to metabolize glucose under aerobic or anaerobic conditions; it also provides protection against oxidative stress (Berlage and Downs. 1996; Enos- Ishii et al., 2007; Juhnke et al., 1996; Lindqvist et al., 1992; Rohmer et al., 1993; Zhang et al., 2003). Addition of glucose and thiamine to the double layer agar creates an optimal growth condition for *E. coli*, therefore providing greater number of healthy host cells for the propagation of bacteriophage MS2.

Calcium chloride has long been recognized for its ability to transform *E. coli* into competent cells; a process that results in significant increases in the electrical conductivity and permeability of the cell plasma membrane. *E. coli* cells treated with $CaCl_2$ are thus more susceptible to phage infection. Calcium supplementation has been intensively used in plaque assays for various bacteriophages; it's suggested that some phages require calcium for adsorption onto the host bacteria. However, even slight excess heating of the medium in preparation may precipitate the calcium so that there is insufficient calcium available for *E. coli* or bacteriophage MS2 (Chung et al., 1989; Cohen et al., 1972; Dagert et al., 1974; Mandel and Higa, 1970; Wenworth, 1963). The heat sensitivity of calcium ion explains why the protocol given by

American Type Culture Collection (2010) instructed addition of filter sterilized $CaCl_2$ solution to the cooled agar instead of autoclaving $CaCl_2$ with the agar. The addition of $CaCl_2$ increases the opportunity for MS2 to bind, penetrate and infect *E. coli*.

The optimized plaque assay displayed superiority over methods described in other publications. When compared to "pouring" methods that mix the phage suspension with the agar (Cronax et al., 1990; Lillehaug, 1997; O'Connell, 2006), this assay was not as easy to handle; care needs to be taken while applying the phage suspension onto the surface the agar. But the relative difficulty of applying the phage suspension was counterbalanced by the high precision and good visibility of plaques achieved in this assay. When compared to "spotting" methods that spot the phage suspension on top of the agar (American Type Culture Collection, 2010; Lillehaug, 1997), this assay was much easier to handle, and the resulting plaque distribution was much more homogenous.

The preparation of the agar is crucial to the success of the plaque assay. The bottom layer and the top layer agar need to be prepared fresh and not stored for later use. Storing the plates at room temperature would lead to loss of moisture and growth of the *E. coli* culture contained in the top layer agar; while storing the plates at refrigeration temperature would cause condensation and possibly death of the *E. coli* cells. When preparing the top layer agar, the *E. coli* culture needs to be seeded at its exponential growth phase instead of stationary growth phase, since cells at exponential growth stage are more susceptible to bacteriophage infection. The bottom agar needs to be dried before the top agar is poured on top, because condensation on the bottom agar would cause the top layer to slide and even fall off. The top layer also needs to be dried in order to prevent excess fluid from interfering with plaque formation. However, the top layer should not be

dried under blowing air for more than 5 min; too much time under the blowing air would cause the surface of the agar to over-dry and make it difficult to spread bacteriophage suspension on top.

The temperatures at which the plates are prepared are also critical to a successful plaque assay. Both layers of agar are to be boiled for sterilization; however, autoclave is not recommended, as loss of moisture during autoclave could alter the composition of the agar and thus affect the outcome of the plaque assay. Heat sensitive ingredients such as glucose, CaCl2, thiamine and *E. coli* 6 h culture should be added to the agar after its temperature has dropped to ~50°C. The temperature at which the agar is solidified also plays an important role in the plaque assay performance. Observations from experiments conducted throughout this study suggested that the top agar should be let solidified at temperatures below 20°C; warmer temperature would cause incomplete solidification. As a result, the gel strength of the top agar would be very weak; just by spreading of bacteriophage suspension would cause the top layer to fall off.

The optimized plaque assay demonstrated improved plaque visibility and plaque count precision. Not only did the optimized assay produce significantly higher plaque count, but also the overall variation between replicates was also much lower than other methods. Although this study was performed on MS2 and its host bacteria *E. coli*, the use of spread plate technique in the plaque assay could be used to enumerate other lytic phages as well. Culture media and host bacteria strains could be modified to suit specific phage types, but the general principal of using spread plate technique and reducing agar thickness and hardness could potentially improve the visibility and accuracy of the plaque assay. The application of the optimized plaque assay would improve the accuracy of phage enumeration and be especially helpful for PCR standard curve construction.

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Chapter 4. Concentration and detection of enteric virus indicator from seawater using activated carbon

Part I: Concentration of bacteriophage MS2 from artificial seawater with adsorbents

4.1 Introduction

Infection with hepatitis A virus (HAV) is the leading cause of clinically apparent viral hepatitis in the United States. HAV infection is primarily transmitted via the fecal-oral route (Centers for Disease Control and Prevention, 2011; Mast and Alter, 1993), and outbreaks of HAV due to consumption of raw or inadequately cooked shellfish harvested from sewage-polluted waters have been documented (Desenclos et al., 1991; Halliday et al., 1991). HAV is often detected in shellfish, due to the ability of shellfish to actively bio-concentrate HAV as much as 100 fold from fecal contaminated waters (Le Guyader et al., 2012; McLeod et al., 2009; Nappier et al., 2008; Wang et al., 2008).

Current techniques for detection of HAV are usually expensive and time-consuming for routine screening (Atmar et al., 1995; Lees et al., 1995). Therefore, a suitable indicator organism is usually used in the routine screening of the presence of HAV. Male-specific (FRNA) bacteriophages have been proposed as indicators of enteric viruses. The abundance of these phages in sewage, their genomic and physical resemblance of HAV, and the ease with which they can be enumerated make them attractive indicators of viral contamination in the environment (Havelaar et al., 1993). Bacteriophage assay conditions are much simpler and cheaper than the enteric virus detection methods. As a group I male-specific RNA coliphage that infects *Escherichia coli* (Nappier et al., 2008), MS2 is used widely as a surrogate for enteric viruses, and its presence has been proven to be a reliable indication of the presence of HAV (Allwood et al., 2003; Huertas et al., 2003; Shin et al., 2003; Tree et al., 2003; Katz and Margolin, 2007).

Numbers of viruses in contaminated food or water are usually too low for detection, however, even one infective HAV is sufficient to cause hepatitis A infection in humans (Cuthbert, 2001). Therefore, large volumes of water (10-20 L) need to be concentrated before analysis can be carried out. Because of the small size of viral particles, mechanical filtration is often not possible; therefore, adsorption-elution methods are employed. These involve manipulation of charges on the virus surface, using pH changes to maximize their adsorption to charged filters (Fong and Lipp, 2005; Lipp et al., 2001; Pallin et al., 1997). However, electropositive filters are easily clogged and have low recovery rates for viruses in marine water due to the interference of salt (Lukasik et al., 2000); while the recovery of viruses from an electronegative filter is not always easy (Fong and Lipp, 2005; Sobsey et al., 1973; Sobsey, 1995). Ultrafiltration methods utilize a flow pattern to force water through a cylindrical filter with pressure while keeping and retaining particles from filters to avoid clogging, and are alternatives to adsorption-elution techniques (Fong and Lipp, 2005; Griffin et al., 2003; Paul et al., 1991). However, ultrafiltration methods are more costly and time-consuming than adsorption-elution because of the high cost of equipment and limitations on the volume of sample that can be concentrated at one time. Concentrated or eluted water samples usually are further concentrated and purified to reduce the final volume of samples to 1 or 2 ml for processing (Fong and Lipp, 2005; Haramoto et al., 2004; Jiang et al., 2001; Katayama et al., 2002; Lipp et al., 2001; Noble and Fuhrman, 2001). Procedures of secondary concentration have often been reported to cause inhibitory effects in PCR assays (Arnal et al., 1999; Fong and Lipp, 2005).

In an effort to solve many of the disadvantages associated with those methods and come up with one that would reduce the time and cost of virus concentration, the use of granular adsorbent was investigated. The ideal absorbent can be used to concentrate suspended virus particles from seawater, and a small volume (1 ml) of eluent can be used to release adsorbed virus, thus

effectively reducing the sample volume to a minimum without a secondary concentration step. Amberlite™ IRA 900 Cl resin (Polysciences Inc.) is a macroreticular polystyrene type 1 strong base anion exchange resin containing quaternary ammonium groups and its presence in the water ensures removal of anions and weakly associated ions. Activated charcoal is commonly used to remove unwanted colored impurities during recrystallization procedures in chemical processing and its large surface area (500~1400 m^2/g) grants sufficient adsorption (Cheremisinoff and Ellerbusch, 1978). Purigen®, HyperSorb™, Matrix™ and Matrix Carbon™ (SeaChem Laboratories Inc. Madison, GA) are synthetic adsorbents with exceptional organic removal capabilities. Purigen® and HyperSorb™ are macroporous synthetic polymers that remove impurities from water, while Matrix™ is a highly porous media for biofiltration. Matrix Carbon™ is a bead shaped activated carbon for optimal hydrodynamics. This report compares the abilities of different commercially available adsorbents to adsorb bacteriophage MS2 from artificial seawater and selects the optimal adsorbent for the development of a virus concentration method.

4.2 Materials and methods

4.2.1 Bacteriophages and host cells

E. coli (ATCC 15597) was grown at 37°C for 6 h. The growth media of *E. coli* contained 10 g tryptone, 1 g yeast extract, 8 g NaCl per liter of medium. After the medium was cooled to 50°C, the following supplements glucose (0.1%), $CaCl_2$ (2 mmol ml^{-1}) and thiamine (10 µg ml^{-1}) were added to the cooled medium. Bacteriophage MS2 (ATCC 15597-B1) was propagated in the exponentially growing *E. coli* culture overnight (16 h) at 37°C.

4.2.2 Plaque assay

The titer of the MS2 culture was determined by plaque assay. Briefly, 10 ml of bottom agar (1.2%) was poured into 100×15 mm Petri dishes and 8 ml agar (0.6%) containing *E. coli* 6 h culture was poured on top. Supplements of glucose, $CaCl_2$ and thiamine were added to both layers at final concentrations of 0.1%, 2 mM and 10 µg/ml. Thirty microliters of MS2 dilutions were then plated on top of the agar with a hockey stick (bent glass rod). All plates were incubated at 37°C overnight before examined for plaques.

4.2.3 Extraction of viral RNA

RNA was extracted from MS2 suspension using QIAamp Viral RNA mini Kit (Qiagen Inc., Valencia, CA). One hundred forty microliters of MS2 suspension was incubated with 560 µl of lysis buffer (QIAamp Viral RNA mini kit) for 10 min at room temperature. Absolute ethyl alcohol (560 µl) was added to the lysate and mixed by vortexing. The mixture was transferred to a spin column in 630 µl aliquots. The column was washed twice, and the RNA was eluted with 30 µl of RNase-free water containing 0.04% sodium azide and used in PCR immediately.

4.2.4 Real-time PCR (qPCR)

PCR was performed in a total volume of 25 µl with a SmartCycler (Cepheid, Sunnyvale, CA). In each reaction, 10.8 µl of RNA was added to 14.2 µl of mixture containing 0.2 µl 125×RT Enzyme Mix (ArrayScript™ UP Reverse Transcriptase, RNase inhibitor), 12.5 µl 2×RT-PCR Mix (SYBR® Green I dye, AmpliTaq Gold® DNA Polymerase, Ultra-pure, dNTPs, ROX™ passive reference, Optimized buffer components), 0.2 mM of each primer, 10 U RNase inhibitor (Life Technologies, Carlsbad, CA). PCR thermocycling condition was as follows (O'Connell et al., 2006): 48°C for 30 min, 95°C for 10 min, 40 cycles of (95°C for 15 s, 60°C for 1 min), then 95°C for 1 min, and 65°C for 2 min. The primers were as follows: forward: 5' GTC GCG GTA ATT GGC GC 3'; reverse: 5' GGC CAC GTG TTT TGA TCG A 3'.

4.2.5 Standard curve

Standard curve was performed by testing 10-fold dilutions of MS2. The RNA was extracted from 10^{0}-10^{-9} MS2 dilutions as described above. The PCR standard curve was constructed from quantifying all the RNA samples in one reaction, and the cycle threshold (Ct) value of each dilution amplified by PCR were plotted as a function of the logarithm of the starting quantity of phages in plaque forming units (PFU) (Jothikumar et al., 1998; Shin and Sobsey, 2003; Ogorzaly and Gantzer, 2006; Langleta et al., 2007; Bae and Schwab, 2008; Blaise-Boisseau et al., 2010; Furiga et al., 2011; Liu et al., 2011).

4.2.6 Evaluation of different adsorbents

Plaque assay and PCR were used to evaluate the efficiencies of different adsorbents to adsorb MS2 from seawater. A variety of materials including Amberlite™ IRA 900 Cl resin (Polysciences Inc. Warrington, PA), activated charcoal powder (kindly supplied by Dr. Alfonso Davila from Louisiana State University), Purigen®, HyperSorb™, Matrix™, and Matrix Carbon™ (SeaChem Laboratories Inc. Madison, GA) were tested. Briefly, artificially seawater of ~30 parts per thousand (ppt) were made by dissolving SeaChem marine salt (SeaChem Laboratories Inc. Madison, GA) in distilled water. For each test, an experiment group and a negative control group were included. In the experimental group, 500 ml of seawater was spiked with 6~7 log PFU of MS2, and 140 µl of water sample was drawn for RNA extraction; 1 g of adsorbent was then suspended in the seawater with constant agitation at room temperature for 2 h, after which another 140 µl of water sample was drawn for RNA extraction. In the negative control group, 500 ml of seawater was spiked with 6~7 log PFU of MS2 and agitated at room temperature for 2 h. One hundred microliters of sample was drawn every 15 min from both groups, and 30 µl of each sample was plated in the plaque assay in duplicates. The quantity (PFU) of MS2 is expected to

decrease over a period of 2 h in the experiment group, and remain unchanged in the negative control group. To confirm that the decrease of MS2 was due to adsorption by the adsorbent and not by other factors, RNA was extracted from the adsorbent and qPCR was performed. The quantity of MS2 adsorbed by the adsorbent was calculated from the standard curve.

4.2.7 Extraction of viral RNA from adsorbent

After 2 h of constant agitation, the adsorbent was separated from the artificial seawater and incubated with 1.2 ml viral lysis buffer (QIAamp Viral RNA mini kit) for 10 min at room temperature. Absolute ethyl alcohol (1.2 ml) was added to the lysate and mixed by vortexing. The mixture was transferred to a spin column in 630 µl aliquots. The column was washed twice, and the RNA was eluted with 30 µl of RNase-free water containing 0.04% sodium azide and used in PCR detection immediately.

4.3 Results

4.3.1 Amberlite™ IRA 900 Cl resin

Amberlite™ IRA 900 Cl resin was unable to concentrate MS2 from artificial seawater. The plaque assay indicated no significant differences between the experiment and negative control; the level of MS2 remained unchanged in both groups, and the expected reduction of MS2 over time in the experiment group was not observed (Fig 4.1). In addition, RNA extracted from Amberlite™ had no amplification in qPCR in repeated trials, indicating no MS2 was adsorbed.

Amberlite™ IRA 900 Cl resin was further tested in fresh water and artificial seawater of low salinities (10~20 ppt) and high pH (10-12). RNA extracted from the resin in all these conditions had no amplification in qPCR, indicating the resin wasn't able to adsorb MS2 from any of the water. The resin was further enhanced by overnight soaking in alkaline solution (1 M

NaOH) followed by rinsing with distilled water, and then tested in water of 0~20 ppt salinity. qPCR still indicated no positive signal from the resin.

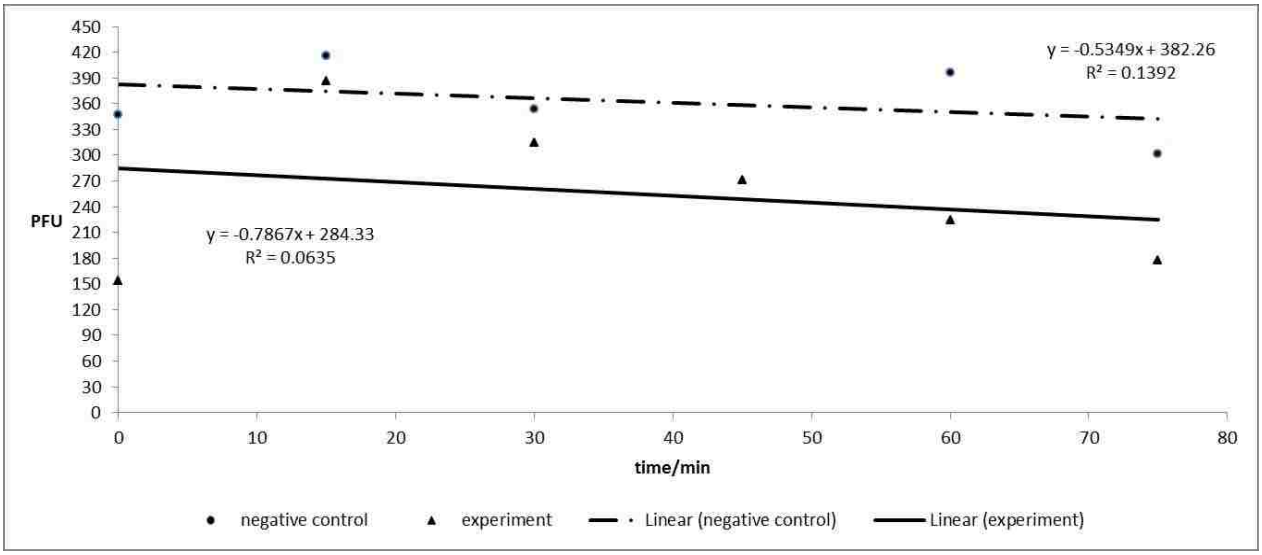

Fig. 4.1 Adsorption-time curve of MS2 by Amberlite™ IRA 900 Cl resin.

4.3.2 Purigen®

Interestingly, RNA extracted from Purigen® showed positive amplification in qPCR. When 7 logs of MS2 were inoculated, 5 logs were detected from Purigen®. It appeared that Purigen® adsorbed a significant amount of MS2 from the seawater, however, qPCR detected equal levels of MS2 (5 logs) in the 140 µl of water sample with Purigen® and in the water sample (140 µl) without Purigen®. Therefore it was likely that the MS2 detected from Purigen® was not from the microscopic pores of Purigen®, but rather from the seawater that remained on the surface of the Purigen® granules.

4.3.3 HyperSorb™ and Matrix™

Tests performed on HyperSorb™ and Matrix™ had similar outcome as that of Purigen®. While RNA extracted from both adsorbents showed positive amplification in qPCR, the levels of MS2 in water samples before and after the adsorbents were added only differed by less than 1 log. Thus it was likely that MS2 was not adsorbed by either of these adsorbents.

4.3.4 Activated charcoal powder

Activated charcoal powder (kindly provided by Dr. Alfonso Davila from Louisiana State University) showed promising adsorption ability. When 8 logs of MS2 were inoculated into 20 ml of artificial seawater, 6 logs were detected in the water sample (140 µl) without activated charcoal, and none was detected in the water sample with activated charcoal. Therefore, it was concluded that activated charcoal removed all the MS2 from the spiked seawater. However, handling activated charcoal powder was extremely challenging due to its fine size (0.15-0.25 mm). Filter paper of pore size 20-25 microns were used to separate charcoal powder from the seawater, however, attempts at isolating RNA off the powder failed in repeated trials.

4.3.5 Matrix Carbon™

Matrix Carbon™ was effective in adsorbing MS2 from seawater. PCR showed that the level of MS2 in the water sample with Matrix Carbon™ was 3 logs lower than that in the water sample without Matrix Carbon™. Plaque assay further confirmed this observation. The level of MS2 in the experiment group (500 ml seawater spiked with 6~7 log PFU of MS2 with 1 g granular activated carbon) exhibited a linear drop over a period of 165 min while the level remained the same in the negative control group (500 ml seawater spiked with 6~7 log PFU of MS2 without granular activated carbon). The adsorption of MS2 onto granular activated carbon exhibited a time-dependent rate of roughly 3.8 log PFU/min with an R^2 value of 0.957 (Fig 4.2).

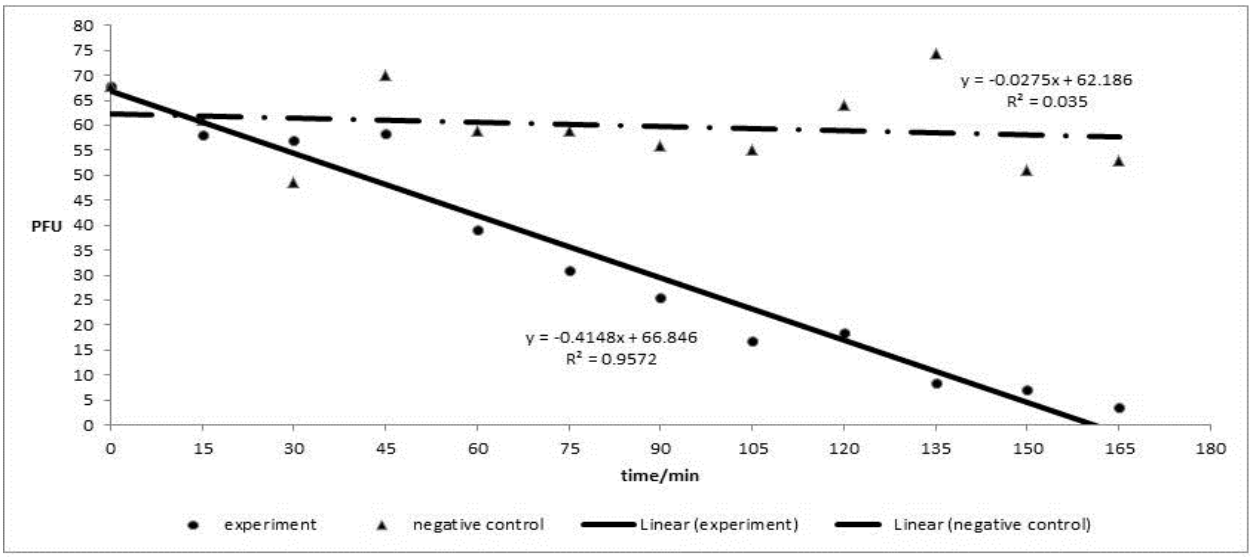

Fig. 4.2 Adsorption-time curve of MS2 by Matrix Carbon™.

4.4 Discussion

The use of a granular adsorbent to adsorb bacteriophage MS2 from artificial seawater showed promising potential for this approach to be applied to virus concentration from seawater. The selection of an optimal adsorbent for MS2 concentration was rather interesting. Granular activated carbon (Matrix Carbon™) was significantly more efficient in concentrating MS2 from artificial seawater over other commercially available adsorbents. While adsorbents with ion-exchange abilities such as Amberlite™ IRA 900 Cl resin, Purigen®, HyperSorb™ and Matrix™ failed to adsorb MS2 even from small volumes of seawater (20 ml), granular activated carbon was able to concentrate 6 logs of MS2 from 500 ml of seawater.

The inability of ion-exchange resins to attract MS2 from seawater could stem from the interference of high level chloride ions in the seawater. As weak electrolytes, the viral particles could be outcompeted by strong electrolytes (chloride) and were not able to attach to the surface of the ion-exchange resins. Our various attempts at modifying the surface charge of the resins and

increasing their abilities to adsorb MS2 by pre-soaking the resins in alkaline solutions, increasing the pH of the seawater, and adjusting the salinity of the water all failed to enhance the adsorption.

The porous structure of granular activated carbon (Matrix Carbon™) granted maximized surface/volume ratio (600 m^2/cc) and was optimal for particle adsorption. The adsorption-time curve revealed a linear relationship between time of contact and quantity of MS2 adsorbed by the activated carbon. It was suggested that the adsorption occurred via short-distance attraction, and could potentially involve forces such as Van der Waals force or hydrogen bond. The constant agitation of the seawater provided maximum chance of contact. Interestingly, RNA extracted from the activated carbon had negative PCR amplification during repeated trials. Since both the plaque assay and PCR proved that MS2 was adsorbed by the activated carbon, it was concluded that the RNA extraction method using viral lysis buffer containing guanidine thiocyanate was unable to release bound MS2 from the inner cavity/pores of activated carbon granules, and that the attraction between MS2 and the activated carbon might be stronger than molecular force, and thus a different eluent was required to elute MS2 off the activated carbon.

Activated carbon powder (kindly provided by Dr. Alfonso Davila from Louisiana State University) was even more efficient in adsorbing MS2 from seawater than granular activated carbon (Matrix Carbon™), leaving no trace of MS2 in the water after the powder was suspended in the seawater. However, the fine size (0.15-0.25 mm) of the powder proved to be problematic to detection; not only was it difficult to separate the powder from the seawater afterwards, it was almost impossible to elute the adsorbed MS2.

The use of plaque assay in combination of qPCR provided solid evidence in the ability of different adsorbents to remove suspended viral particles from water. The plaque assay supplied additional information regarding the speed and linearity of the removal. Although less costly than

PCR, several drawbacks of plaque assay makes it less suitable than qPCR. Plaque assay requires at least 12 h before results could be observed, while PCR only requires 2 h; the accuracy of plaque assay is dependent on the preparation of the agar plates as well as the experience of the handler; host bacteria culture needs to be freshly grown for more reliable outcome; agar plates used in the plaque assay can be subject to contamination; only plaque numbers between 20 to 200 could be quantified. Therefore, the assessment of different adsorbents mostly relied on qPCR.

Granular activated carbon (Matrix Carbon™) was shown to be effective in adsorbing bactgeriophage MS2 from artificial seawater and has the potential to be used in virus concentration and detection from shellfish growing waters.

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Part II: Effect of temperature, pH, and salinity on the concentration efficiency of granular activated carbon

4.6 Introduction

Current virus concentration methods are often time-consuming, expensive, and require additional concentration steps. In an effort to solve many of the disadvantages associated with these methods and reduce the time and cost, a virus concentration method using granular adsorbent was developed. The ideal absorbent can be used to concentrate suspended virus particles from seawater, and a small volume (1 ml) of eluent can be used to elute adsorbed virus, thus effectively reducing the sample volume to a minimum without a secondary concentration step. In the preliminary study, granular activated carbon (Matrix Carbon™) showed promising potential in concentrating high levels of bacteriophage MS2 from artificial seawater. Activated carbon consists of a wide range of amorphous carbonaceous materials with a high degree of porosity and extended inter-particulate area obtained by combustion, partial combustion, or thermal decomposition (Bansal and Meenakshi, 2010).

Activated carbon exists in granular or powder forms. The granular from has a large internal surface area and small pores while the finely divided powder form is associated with larger pore diameters and smaller internal surface area. Granular activated carbon is commonly used in water reclamation for organic matter removal, and the pore-size distribution of granular activated carbon was revealed to be the key contributor to the adsorption capacity and rate of uptake of contaminants (Lee et al., 1981). Various studies suggested the potential of activated carbon in virus adsorption from waste water (Cookson and Wheeler. 1967; Gerba et al., 1975; Powell et al., 2000). Activated carbon was documented to remove 99% of viruses from the water, and adsorb up to 12 logs of viruses per gram, while the adsorbed virus maintains infectivity (Cookson and Wheeler. 1967; Powell et al., 2000). As no previous studies have conducted their experiment in seawater,

this report compares fresh water with seawater, and investigated the effects of different parameters of the seawater (temperature, salinity and pH) on the concentration efficiency of granular activated carbon.

4.7 Materials and methods

4.7.1 Bacteriophages and host cells

E. coli (ATCC 15597) was grown at 37°C for 6 h. The growth media of *E. coli* contained 10 g tryptone, 1 g yeast extract, 8 g NaCl per liter of medium. After the medium was cooled to 50°C, the following supplements glucose (0.1%), $CaCl_2$ (2 mmol ml^{-1}) and thiamine (10 µg ml^{-1}) were added to the cooled medium. Bacteriophage MS2 (ATCC 15597-B1) was propagated in the exponentially growing *E. coli* culture overnight (16 h) at 37°C.

4.7.2 Viral titration

The titer of the MS2 culture was determined by plaque assay. Briefly, 10 ml of bottom agar (1%) was poured in 100×15 mm Petri dishes and 10 ml agar (0.45%) containing *E. coli* 6 h culture was poured on top. Supplements of glucose, $CaCl_2$ and thiamine were added to both layers at final concentrations of 0.1%, 2 mM, and 10 µg/ml. Thirty microliters of MS2 dilutions were then spread on top of the agar with a hockey stick. All plates were incubated at 37°C overnight before examined for plaques.

4.7.3 Real-time PCR (qPCR)

PCR was performed in a total volume of 25 µl with a SmartCycler (Cepheid, Sunnyvale, CA, USA). In each reaction, 10 µl of RNA was added to 15 µl of mixture containing 0.2 µl 125×RT Enzyme Mix (ArrayScript™ UP Reverse Transcriptase, RNase inhibitor), 12.5 µl 2×RT-PCR Mix (SYBR® Green I dye, AmpliTaq Gold® DNA Polymerase, Ultra-pure, dNTPs, ROX™ passive reference, Optimized buffer components), 0.2 mM of each primer, 1.5 µl RNase-free

water. PCR thermocycling condition was as follows (O'Connell et al., 2006): 48°C for 30 min, 95°C for 10 min, 40 cycles of (95°C for 15 s, 60°C for 1 min), then 95°C for 1 min, and 65°C for 2 min. The primers were as follows: forward: 5' GTC GCG GTA ATT GGC GC 3'; reverse: 5' GGC CAC GTG TTT TGA TCG A 3'.

4.7.4 Standard curve

Standard curve was performed by testing 10-fold dilutions of viral solution of MS2. The RNA was extracted from 10^{0}-10^{-9} MS2 dilutions using QIAamp Viral RNA mini Kit (Qiagen Inc., Valencia, CA). The PCR standard curve was constructed from quantifying all the RNA samples in one reaction, and the cycle threshold (Ct) value of each dilution amplified by PCR were plotted as a function of the logarithm of the starting quantity of phages in PFU (Jothikumar et al., 1998; Shin and Sobsey, 2003; Ogorzaly and Gantzer, 2006; Langleta et al., 2007; Bae and Schwab, 2008; Blaise-Boisseau et al., 2010; Furiga et al., 2011; Liu et al., 2011). The quantity of phages can also be expressed in genome copies (Dreir et al., 2005; Rolfre et al., 2007; Pecson et al., 2009; Dong et al., 2010). Rolfe and others (2007) calculated the genome copy number of MS2 to be 9.18 per PFU (Rolfe et al., 2007).

4.7.5 Concentration and elution

Each step of the concentration and elution methods is shown in Fig 4.3. Artificial seawater (10, 20, 30 and 40 parts per thousand, ppt) were made from SeaChem Marine Salt (SeaChem Laboratories Inc. Madison, GA). Fresh water of pH 4-9 were made from 10 mM analytical grade buffers (citric buffer, pH 4, 5, and 6; phosphate buffer, pH 7; Tris-HCl buffer, pH 8 and 9). Five hundred milliliters of water samples were stored at temperatures 4, 20 and 37°C overnight before inoculated with 9 log PFU of MS2. One gram of granular activated carbon (Matrix Carbon™, SeaChem Laboratories Inc. Madison, GA) was suspended in the spiked water for 3 h under

uniform temperature. To elute the adsorbed viruses from the activated carbon, 1 ml of different eluents (0.25% trypsin-EDTA solution, Sigma-Aldrich Co. St. Louis, MO); sodium dodecyl sulfate (2%, 5% and 10%); viral lysis buffer (QIAamp Viral RNA mini Kit)) were used for elution and their efficiencies in recovering the viruses were compared.

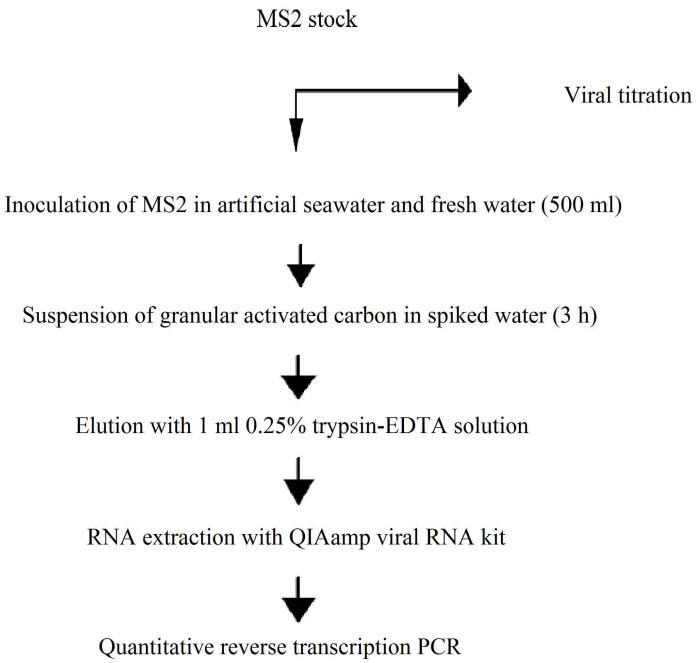

Fig. 4.3 Protocol for concentration of bacteriophage MS2 from artificial seawater.

4.7.6 Extraction of viral RNA from eluate

After 2 h of incubation, the eluents were separated from the activated carbon and used for RNA extraction. Every 140 µl of the eluent was incubated with 560 µl of lysis buffer (QIAamp Viral RNA mini kit) for 10 min at room temperature. Absolute ethyl alcohol (560 µl) was added to the lysate and mixed by vortexing. The mixture was transferred to a spin column in 630 µl aliquots. The column was washed twice, and the RNA was eluted with 30 µl of RNase-free water containing 0.04% sodium azide and used in PCR immediately.

4.7.7 Comparison of different parameters of seawater

The effects of salinity, pH and temperature on the ability of granular activated carbon to concentrate MS2 were tested. Nine log PFU of MS2 were inoculated into 500 ml of artificial seawater of different salinities, pH and temperatures and concentrated with 1 g of granular activated carbon. The viruses were eluted with 1 ml of trypsin-EDTA solution (0.25%) and RNA was extracted from the eluate and used for qPCR. The quantity of MS2 detected by qPCR was used as a basis for comparison. The experiments were done in quadruplicate.

4.7.8 Statistical analysis

The effects of salinity, temperature and pH on the concentration efficiency were analyzed statistically by comparing the mean PFU of MS2 detected under each condition. Pairwise comparisons were carried out with Tukey's HSD by JMP (SAS Institute Inc. Cary, NC, USA).

4.7.9 Virus concentration from natural seawater

Samples of natural seawater near oyster beds were taken from Bay St. Louis, Mississippi in January of 2011. Five hundred milliliters of samples were stored at 20 and 37°C and inoculated with 9 logs of MS2. Concentration and elution were performed as described previously.

4.8 Results

4.8.1 Elution of MS2 from granular activated carbon

In the comparative study of different elution buffers, it was observed that trypsin-EDTA solution (0.25%) had a much higher efficiency of eluting MS2 from granular activated carbon when compared to other buffers, since all the other buffers were completely unable to elute MS2 (data not shown). This eluent was therefore selected in the following experiments.

4.8.2 Comparison of different parameters of seawater

Granular activated carbon exhibited different efficiencies in concentrating MS2 under different conditions. Its efficiency significantly increased with warmer temperature; at each salinity, activated carbon was able to adsorb 10-fold more MS2 at 37°C than at 20°C, and 100-fold more than at 4°C. It also concentrated more MS2 from acidic buffers than from basic buffers. The efficiency was also much higher in seawater than in fresh water at all three temperatures; on average granular activated carbon was able to adsorb 10-fold more MS2 from artificial seawater than from acidic buffers, and 100 to 1000-fold more than from basic buffers (Table 4.1 and 4.2).

Table 4.1 Effects of pH and temperature on the concentration efficiency of activated carbon [a]

pH	Temperature (ºC)	Log PFU of released MS2 [b]
4.0	4	$3.1 \pm 0.3^{B, C, D, E}$
	20	$4.2 \pm 0.7^{A, B, C, D}$
	37	4.8 ± 0.5^{A}
5.0	4	2.6 ± 0.5^{E}
	20	$3.9 \pm 0.5^{A, B, C, D, E}$
	37	5.0 ± 0.5^{A}
6.0	4	$4.0 \pm 0.6^{A, B, C, D, E}$
	20	$4.6 \pm 0.3^{A,B,C}$
	37	$4.6 \pm 0.4^{A, B}$
7.0	4	$2.8 \pm 1.0^{D, E}$
	20	$3.1 \pm 0.9^{B, C, D, E}$
	37	$4.1 \pm 0.4^{A, B, C, D, E}$
8.0	4	2.6 ± 0.7^{E}
	20	ND
	37	2.6 ± 0.8^{E}
9.0	4	$3.0 \pm 0.8^{C, D, E}$
	20	ND
	37	ND

[a] An initial inoculation of 9± 0.8 log pFU was used in all the conditions.
[b] Means based on 4 replicates were compared using Tukey's HSD (JMP software). Means within each column followed by at least one same uppercase letter are not significantly different ($P > 0.05$).

4.8.3 Statistical analysis

The effects of different parameters of seawater on the concentration efficiency were rather interesting. Temperature had a significant impact on the concentration efficiency, regardless of the salinity of water. The activated carbon adsorbed significantly more MS2 at 37°C than at 20°C, and

even more so than at 4°C. The influence of temperature also skewed the impact of pH and salinity. At 4°C, the change in pH did not contribute to significantly different concentration efficiencies; however, when the temperature of the water was raised beyond 20°C, the difference between acidic buffers and basic buffers became significant (Table 4.1). The effect of salinity was also influenced by temperature. At 4°C, there was no significant difference between fresh water and seawater; at 20°C, the difference between basic buffers and seawater became signifianct, and at 37°C, the concentration efficiency in seawater was significantly higher than all the fresh water (Table 4.2). The efficiency in seawater of different salinities also varied: granular activated carbon adsorbed ~0.3 log PFU more MS2 from seawater of low salinity (10 ppt and 20 ppt) than high salinity (30 ppt and 40 ppt). At its peak performance (20 ppt seawater at 37°C), granular activated carbon was able to concentrate 6.4 log PFU MS2 from 500 ml seawater (Fig 4.4).

Table 4.2 Effects of salinity and temperature on the concentration efficiency of activated carbon [a]

Salinity (ppt)	Temperature (ºC)	Log PFU of released MS2 [b]
10	4	4.1 ± 0.4^{F}
	20	$4.9 \pm 0.7^{D, E, F}$
	37	$6.2 \pm 0.2^{A, B}$
20	4	4.1 ± 0.5^{F}
	20	$5.2 \pm 0.2^{C, D, E}$
	37	6.3 ± 0.3^{A}
30	4	$4.3 \pm 0.7^{E, F}$
	20	$5.2 \pm 0.4^{B, C, D, E}$
	37	$5.8 \pm 0.3^{A, B, C, D}$
40	4	$4.2 \pm 0.4^{E, F}$
	20	$5.3 \pm 0.2^{A, B, C, D}$
	37	$6.0 \pm 0.3^{A, B, C}$

[a] An initial inoculation of 9± 0.8 log pFU was used in all the conditions.

[b] Means based on 4 replicates were compared using Tukey's HSD (JMP software). Means within each column followed by at least one same uppercase letter are not significantly different ($P > 0.05$).

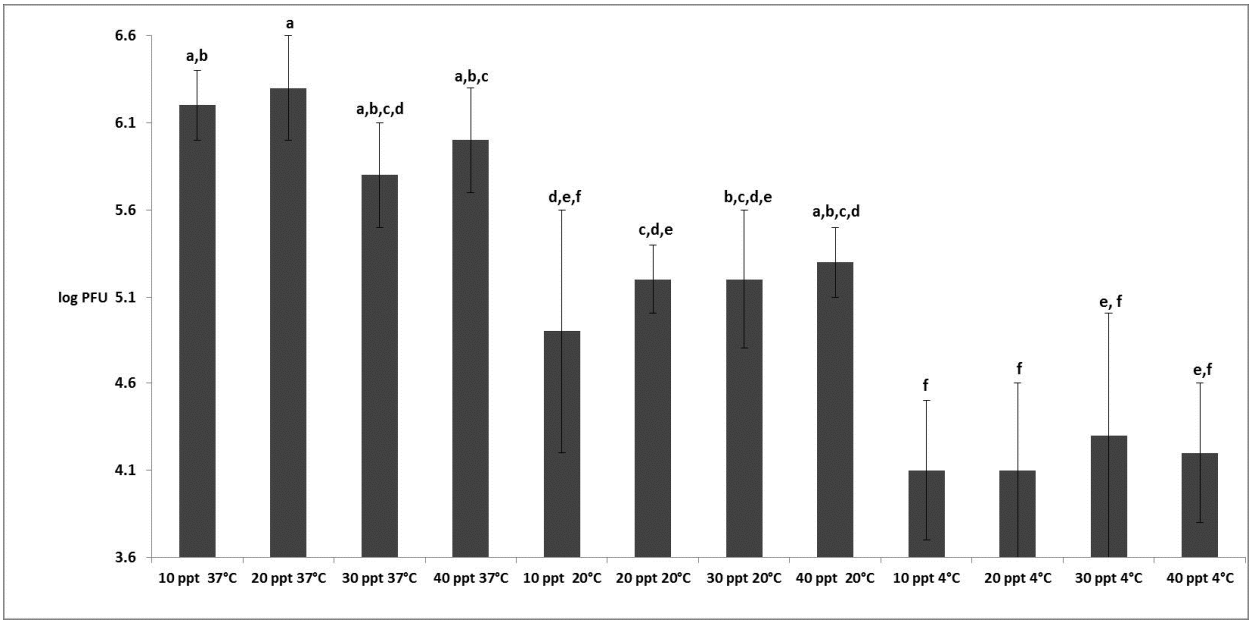

Fig. 4.4 Quantity of MS2 concentrated by granular activated carbon under different salinities and temperatures. The statistical connecting letters were displayed under each condition.

4.8.4 Virus concentration from natural seawater

The natural seawater sampled from Bay St. Louis had a salinity of approximately 20 ppt. The effect of temperature in natural seawater was similar to that in artificial seawater; the activated carbon adsorbed almost 2 logs more MS2 at 37ºC than at 20ºC (Table 4.3). At 20ºC, activated carbon adsorbed ~0.6 log less MS2 from natural seawater than from artitifical seawater of the same salinity (20 ppt); while at 37ºC, activated carbon was equally efficient in natural seawater and in artificial seawater.

Table 4.3 Virus concentration from natural seawater [a]

Salinity (ppt)	Temperature (ºC)	Log PFU of released MS2 [b]
21.9	20	4.6 ± 0.2
21.4	37	6.4 ± 0.02

[a] An initial inoculation of 8.9± 0.4 log pFU was used in all the conditions.
[b] Two replicates were used in each condition.

4.9 Discussion

It was unclear what types of interaction were involved in the absorption of MS2 by granular activated carbon (Matrix Carbon™). Traditionally, the surface interaction has been contributed to electrostatic force (Cookson and Wheeler, 1967; Gerba et al., 1975; Silva et al., 1996; Powell et al., 2000). However, failure from repeated attempts at eluting MS2 off activated carbon with common eluents suggested that there might have been a stronger bond than weak intermolecular force. The sole success of trypsin solution at releasing MS2 from activated carbon suggested that a protease was necessary in breaking off the attraction, hinting a potential protein bonding between the MS2 capsid protein and the inner cavity of activated carbon. Getzen and Ward (1969) proposed a theoretical model to explain the adsorption of both ionic and molecular species as a function of pH. Their explanation centered on the function of hydrogen ions to attract anions from the water. However, repeated experimentation in this study revealed that the impact of pH on MS2 concentration was only significant at warm temperatures, so it's unclear whether the viral particles were attracted to activated carbon granules via hydrogen ions.

Study by Cookson and Wheeler (1967) indicated no impact of temperature on the performance of activated carbon, as opposed to results observed in this study. It was observed temperature had a significant impact on the concentration efficiency, and its influence also skewed the effects of other parameters such as pH and salinity. It was suggested that the absorption of MS2 by activated carbon occurred via short-distance attraction, and constant agitation of the seawater provided maximum chance of contact. This could explain the role of temperature in the process. As temperature rises, the molecular kinetic energy increases and accelerates the interaction between molecules (McNaught and Wilkinson, 1997), elevating the level of impact between activated carbon granules and suspended MS2, thus enhances absorption of MS2.

The effect of salinity on the efficiency of granular activated carbon was rather interesting. Increasing the salinity from 0 to 10 ppt drastically increased the absorbed MS2 by ~3 logs, and changing the salinity from 10 to 40 ppt resulted in a difference of less than 1 log. It was unclear how the presence of marine salt affected the adsorption of MS2. A possible explanation could be ions from the seawater were attracted onto the surface of the activated carbon and then bridged the absorption of the viral particles onto the activated carbon. The miraculous improvement in seawater as opposed to fresh water would eliminate the need of dialysis and acidification of seawater during virus concentration. In addition, shellfish beds are usually located at estuaries and thrive at salinity of approximately 20 ppt (Marsden, 2004; Jean-Claude et al., 2009). Granular activated carbon was shown to be equally efficient in natural seawater and in artificial seawater of the same salinity (20 ppt) at 37ºC. Therefore, the optimal performance of granular activated carbon in 20 ppt seawater suggested that activated carbon could be used for large-scale concentration and detection of enteric viruses from shellfish harvesting seawater.

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Chapter 5. Concentration of hepatitis A virus from artificial seawater using zeolite

Part I: Optimization of cell infectivity assay and qPCR for detection of HAV

5.1 Introduction

Hepatitis A virus (HAV) is one of the few enteric viruses for which conclusive evidence of waterborne transmission exists, and common source outbreaks have been attributed to fecal contaminated food and drinking water (Rao and Melnick, 1986). A causal association between hepatitis A and the use of fecal contaminated water for recreation purposes has also been proven (Mahoney et al., 1992). Routine screening of the presence of HAV in recreational water and shellfish-growing areas can help monitor the level of contamination and prevent outbreaks of gastroenteritis. Therefore, it's critical to develop reliable methods to detect and quantify HAV.

Although HAV can be elucidated by cell culture, the method is expensive and requires highly specialized equipment. Furthermore, certain cell lines will recover only certain virus type. In addition, molecular techniques for detection of HAV are usually expensive and time-consuming (Atmar et al., 1995; Lees et al., 1995). During the attempted propagation of HAV *in vitro*, we have discovered that current literature lacked a detailed and clear description of the procedures, and that our interpretation of the current methodologies on HAV propagation and quantification often failed to reproduce their results. Because the establishment of a robust procedure for HAV propagation and elucidation is extremely critical to the credibility of research and interpretation of the results, we found it necessary to optimize the cell infectivity assay and qPCR. In an attempt to optimize the propagation, elucidation and molecular detection of cell-adapted HAV, we tested various parameters of cell culture, cell infectivity assay and real-time PCR (qPCR) detection, and their influence on the replication rate and cytopathic effect (CPE) production of HAV in cell culture, and the efficiency as well as accuracy of qPCR.

5.2 Materials and methods

5.2.1 Selection of host cell line

African green monkey kidney cell line (B-SC-1) (ATCC CCL-26) was the original host cells for HAV strain HM175/18f, so it was selected at first for propagation and titer determination of HAV. Alternatively, rhesus monkey kidney cells (FRhK-4) (ATCC CRL-1688) was used for virus propagation due to reports that this cell line produces more obvious CPE (Dotzauer et al., 1994; Mbithi et al., 1990; Romalde et al., 1994). Hepatitis A virus strain HM175/18f (ATCC VR1402) was propagated in both cell lines and the onset of CPE were used as a basis for comparison regarding the suitability of the cell line.

5.2.2 Methods for thawing the host cells

Centrifugation. The frozen culture of the selected monkey kidney cells (ATCC, Manassas, VA) was pulled from liquid nitrogen, immediately placed in 45°C water bath and agitated till thawed. The vial was thoroughly sprayed with 70% ethanol and the content (~1 ml) was added into 3 ml Dulbecco's modified eagles medium (DMEM) (Lonza, Walkersville, MD) with 20% fetal bovine serum (FBS) (Lonza, Walkersville, MD) and centrifuged at 1000 rpm (200 × *g*) for 10 min. The supernatant was discarded and the cell pellet was resuspended with fresh 1 ml 20% FBS DMEM. The cell pellet was homogenized by gentle pipetting and transferred to a T75 flask (Corning Inc., Corning, NY) containing 20 ml 20% FBS DMEM. The cells were incubated at 37°C with 5% CO_2.

Media dilution. Alternatively, a frozen culture of monkey kidney cells was pulled from liquid nitrogen, immediately placed in 45°C water bath and agitated till thawed. The vial was thoroughly sprayed with 70% ethanol and the content was immediately transferred to a T75 flask containing 20 ml of pre-warmed fresh 10% FBS DMEM and incubated at 37°C with 5% CO_2.

5.2.3 Cell culture environment selection

CO_2 incubation. Cells were grown in Dulbecco's modified eagles medium (DMEM) supplemented with serum, and 5% CO_2 was used in the incubator to maintain a balanced pH. Since DMEM contained 3.7g/L sodium bicarbonate, the level of CO_2 was then adjusted to 10% for proper pH maintenance.

CO_2-free incubation. Alternatively, cells were grown in L-15 medium (Sigma-Aldrich Co. St. Louis, MO) supplemented with serum in a CO_2-free incubator. L-15 medium did not contain sodium bicarbonate, thus didn't require the use of CO_2 for pH balance.

5.2.4 Cell passage

Cell scraper detachment. Nunc* cell scraper (Thermo Scientific Co., Waltham, MA) was originally used for cell passage. When cells reached 80~90% confluency, the old cell culture media was discarded, and 10 ml of fresh cell culture media containing 10% serum was added to the cell culture flask. The cells were carefully scraped from the flask and homogenized by gentle pipetting with the addition of 30 ml more cell culture media. Half of the cell culture media containing detached cells was transferred to a new cell culture flask and both flasks were incubated at 37°C.

Trypsin detachment. When cells reached 80~90% confluency, the old cell culture media was discarded, and flasks were rinsed with 3 ml trypsin-EDTA solution (0.25% porcine trypsin) (Sigma-Aldrich Co. St. Louis, MO) followed by mild agitation, then 2 ml trypsin solution was added and the flask was incubated at 37°C for 5~10 min until all the cells were detached. The rinse step was essential for successful cell detachment due to trypsin inhibitors in bovine serum (Twist et al., 1984). Fresh cell culture media containing serum (10 ml) was added and the cells were homogenized by gentle pipetting, followed by addition of 30 ml more fresh cell culture media and half of the cell culture media containing detached cells was transferred to another flask (Fig 5.1).

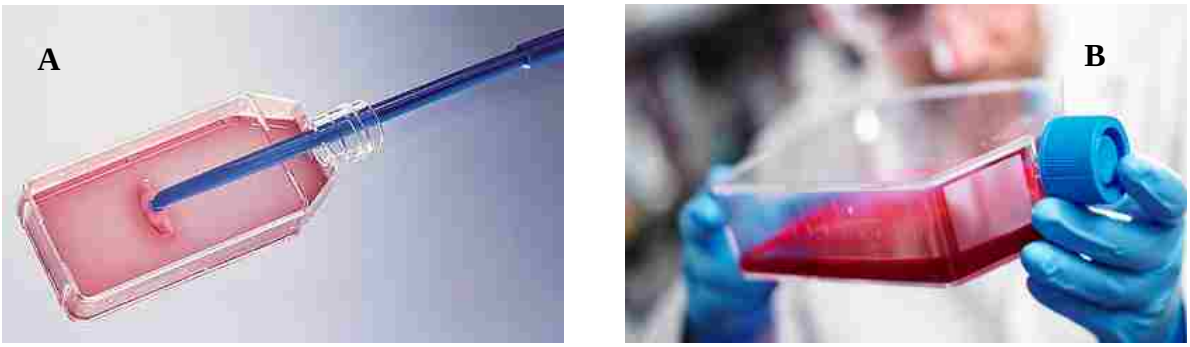

Fig. 5.1 (A) cell scraper detachment (www.coleparmer.com) and (B) trypsin detachment (www.biopharma.novozymes.com)

5.2.5 Virus propagation

High serum propagation. Virus was propagated in the selected cell line. The old cell culture media was replaced with ~24 ml fresh 10% FBS DMEM when cells reach 80%~90% density, then 0.5 ml of hepatitis A virus strain HM175/18f stock was thawed from -80°C and immediately transferred to the cell culture flask. The cells were incubated at 37°C until obvious CPE were observed. The cell culture flask was then frozen at -80°C and thawed and frozen again. The culture was then thawed and the content was homogenized by gentle pipetting and aliquoted into 1 ml cryovials. The cryovials were stored at -80°C.

Low serum propagation. Virus propagation was carried out when host cells reached 80~90% confluency. The old cell culture media was discarded and the cell culture flask (T75) was rinsed twice with serum free cell culture media (DMEM), followed by addition of 4.5 ml serum free cell culture media. Hepatitis A virus strain HM175/18f stock was thawed from -80°C and 0.5 ml of virus stock was immediately transferred to the cell culture flask. The flask was incubated at 37°C and rocked side to side every 15 min for 2 h, afterwards 5 ml more cell culture media was added to the flask, and FBS was added to a final concentration of 2%. The cells were incubated at 37°C until obvious CPE were observed. The cell culture flask was then frozen at -80°C and thawed

and the content was homogenized by gentle pipetting and aliquoted into 1 ml cryovials. The cryovials were stored at -80°C.

5.2.6 Virus titration

Plaque assay with agarose. Plaque assay is commonly used in titer determination of HAV (Blaise-Boisseaua et al., 2010; Dubois et al., 2007; Kingsley and Richards 2001). Plaque assays make use of viscous overlays to cover cells immediately after infection, thus limiting virus spread and restricting virus growth to foci of cells at the sites of initial infection. If viruses induce strong CPE, cells in plaques are lysed and plaques can be visualized by staining of the residual intact cells (Herzog et al., 2008).

Host cells were grown 6-well tissue culture dishes (Corning Inc., Corning, NY) until 80~90% confluency. The cell culture media was discarded and the wells were washed with phosphate buffered saline (PBS) or serum-free media. Serial dilutions of HAV stock were made from PBS or serum-free media. One milliliter of serum-free media was added to each well, and 140 µl of HAV dilution (10^{-5}~10^{-1}) was added to each well. One well of each tissue culture dish was used as negative control in which no virus dilution was inoculated. Meanwhile 5% agarose (Lonza, Walkersville, MD) was melted in boiling water bath and cooled to 45°C. Prewarmed cell culture media and FBS was added to the agarose solution to a final concentration of 0.5% agarose and 5% FBS. After 2 h of incubation, virus dilutions were aspirated from all wells and 4 ml of 0.5% agarose solution was added to each well. The tissue culture dishes were incubated at 37°C after agarose solidified. After 7 days of incubation, 1 ml of 0.03% neutral red solution (Sigma-Aldrich Co. St. Louis, MO) was added to each well and incubated for 1 h at 37°C incubator. The neutral red was then removed and the tissue culture dishes were inspected for plaque formation.

Alternatively, agarose was carefully scraped off the tissue culture dish and the cells were stained with 1% crystal violet solution. One milliliter of crystal violet solution was added to each well and aspirated after 5 min, the wells were then washed with 1 ml of distilled water and inspected for plaque formation.

Plaque assay with carboxymethylcellulose. Plaque assay was also carried out using carboxymethylcellulose instead of agarose. As an alternative to solid gels, viscous solutions of soluble hydrophilic polymers such as carboxymethylcellulose can be employed (Fig 5.2) (Matrosovich et al., 2006). The procedure for plaque assay was similar to the method using agarose as described above, but instead of 0.5% agarose, 1.6% carboxymethylcellulose solution was used to immobilize virus particles. After 2 h of virus inoculation, virus dilutions were aspirated from all wells and 4 ml of 1.6% carboxymethylcellulose solution was added to each well. The tissue culture dishes were incubated at 37°C immediately for 7 days. The carboxymethylcellulose was aspirated after CPE were obvious and 1 ml of 0.03% neutral red solution was added to each well and incubated for 1 h at 37°C incubator. The neutral red was then removed and the tissue culture dishes were inspected for plaque formation.

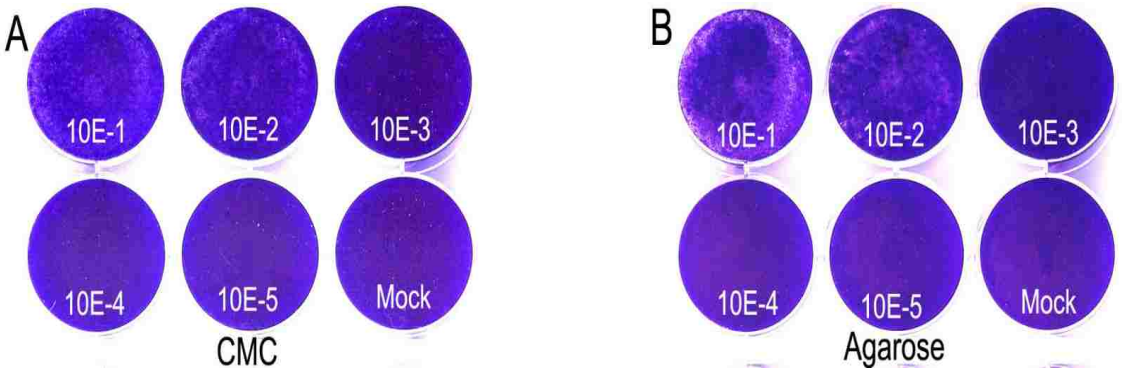

Fig. 5.2 Plaque assay using (A) carboxymethylcellulose and (B) agarose (Herzog et al., 2008)

Tissue culture infectious dose 50% (TCID50) assay. The procedure is performed to determine the infectious titer of any virus which can cause CPE in tissue culture over a reasonable

period of 5 to 20 days while cells in culture remain viable. This procedure is performed to quantify how much infectious virus is in a preparation. Serial 10-fold dilutions were added to multi-well tissue culture dishes and the tissue culture infectious dose 50% (TCID50) was determined on the basis of the number of wells displaying positive CPE (Barrett et al., 1996). Healthy host cells were seeded in eight 6-well tissue culture dishes at 90% confluency and incubated at 37°C for 24 h. Serial 10 fold dilutions of HAV were made in serum-free cell culture media. The 6-well tissue culture dishes were rinsed with serum-free media, and 1 ml of serum-free media and 70 µl of each serial dilution was added to each of ten wells of two 6-well dishes. One well of each dish was used as negative control in which no virus dilution was inoculated. Four serial dilutions were used; each with a 10-fold difference in dilution factor. Thus, in total eight 6-well dishes were used in one TCID50 assay. The plates were incubated at 37°C for 7~10 days, until obvious CPE was seen. The TCID50 was calculated according to Saganuwan using a modified Reed–Muench equation (Saganuwan, 2011).

5.2.6 Traditional qPCR vs. fast qPCR

Traditional qPCR usually take around 2 h to complete (Atmar et al., 1995; Blaise-Boisseaua et al., 2010; Brooks et al., 2005; Costa-Mattioli et al., 2002; Dubois et al., 2007; Houde et al., 2007; Kingsley and Richards, 2001; Traore et al., 1998) while the fast PCR deceloped by Life Technologies can finish in less than 30 min (Protocol: TaqMan® Fast Virus 1-Step Master Mix). To compare the traditional and fast PCR, one standard curve was constructed with the traditional and one with the fast using the same RNA extracts. The thermo cycle conditions representing the traditional PCR were as follows (Dubois et al., 2007): 40 min reverse transcription at 45 °C, followed by 10 min at 95 °C and finally 45 cycles of (15 s at 94 °C and 1 min at 60 °C). The thermo cycle conditions representing the fast PCR were (Protocol: TaqMan® Fast Virus 1-

Step Master Mix): reverse transcription at 50°C for 5 min, initial denaturation at 95°C for 20 s, and 40 cycles of amplification (95°C for 3 s, 60°C for 30 s).

5.3 Results

5.3.1 B-SC-1 cell line vs. FRhK-4 cell line

B-SC-1 cells were the original host cells for HAV strain HM175/18f, so it was selected at first for propagation and titer determination of HAV. However, the B-SC-1 cells did not grow very fast; the doubling time was one to two weeks, and the cells were not very healthy. In addition, no pronounced CPE were observed in the infected cells and no PCR amplifications were achieved from the RNA extracted from the infected cell supernatant. On the contrary, FRhK-4 cells had a doubling time of 2~3 days, and the CPE were obvious after 7 days. PCR amplification of the RNA extracted from infected cell supernatant was seen even at the 10^{-7} dilution. Therefore, FRhK-4 cells were selected for further virus propagation and infectivity assays.

5.3.2 Centrifugation vs. Media dilution

Cryopreserving media usually contain DMSO, which is known to facilitate the entry of organic molecules into tissues and cause cell damage (GIBCO® Cell Culture Basics Handbook; Sigma-Aldrich Cell Culture Manual). Centrifugation (200×g for 10 min) was initially applied to the defrosted cells to remove residual DMSO in the freezing media, according to GIBCO® Cell Culture Basics Handbook. While useful for removing the residual toxins in the freezing media through centrifugation, the cells could be exposed to physical stress during the process and die. The alternative method for thawing the cells was diluting the cells with 25 ml growth media containing 20% serum, according to Sigma-Aldrich Cell Culture Manual. This approach drastically decreased the concentration of residual DMSO surrounding cells and the cells were unharmed. Past experience indicated that cells recover much faster when centrifugation was not applied.

5.3.3 CO_2 incubation system vs. CO_2-free incubation system

Past experience showed that keeping 10% CO_2 tends to cause the connection tube between the gas cylinder and the incubator to blow off, resulting in pH rise in cell culture media and eventually cell death. In addition, the gas cylinder was depleted every two weeks. Due to the inconvenience and raising cost, the incubation system was switched to CO_2-free and the cell culture medium was changed to L-15 medium, a medium without sodium bicarbonate.

5.3.4 Cell scraper vs. trypsin solution

Cell scrapers were originally used for cell passage. It provided a fast and easy procedure for aiding cell detachment. The cell scraper applied physical force to detach cells. However, homogeneity was not easily achieved; the cells growing in the corner of the flask were usually still attached to the wall. Furthermore, cell scrapers can cause physical shear stress to cells and result in more dead cells after passage. Trypsin on the other hand needed more time and more steps for complete cell detachment, but cells were more thoroughly detached, more evenly distributed and healthier after passage. Trypsin treatment was thus chosen as the cell passage method.

5.3.5 High vs. low serum virus propagation

Serum was shown to inhibit virus propagation in host cells. When cell culture media containing 10% serum was used during virus inoculation, CPE was not observed even after one month. In contrast, when serum-containing media was removed and cells were rinsed for residual serum removal during initial virus inoculation, pronounced CPE was observed after only eight days. The observation was consistent with reports on serum inhibition of other viruses (Twist et al., 1984; Qin et al., 2013). In addition, mild agitation during virus inoculation also seemed to speed up virus propagation in host cells and reduced the interval.

5.3.6 Plaque assay vs. TCID50 assay

We were unable to see plaque formation on 6-well tissue culture dishes even when pronounced CPE was observed under the microscope. Despite the clear signs of difference between healthy control cells and dead infected cells (Fig 5.3), no plaques were seen on the culture dish, even with the aid of vital stain such as neutral red (Fig 5.4).

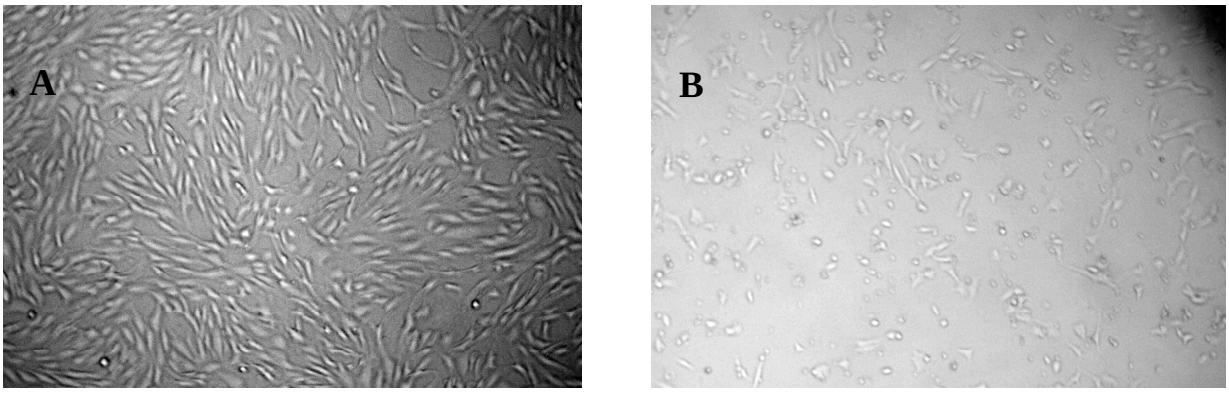

Fig. 5.3 Cytopathic effects of HAV infected FRhK-4 cells (A) control cells; (B) infected cells

Fig. 5.4 Neutral red stained 6-well tissue culture dish 7 days post infection.

It was speculated that agarose diminished the plaque formation and restricted the entry of neutral red. However, attempts at removing agarose and staining cells with crystal violet also proved unsuccessful; despite careful manipulation, some cells were also removed along with

agarose. Replacing agarose with carboxymethylcellulose also failed to produce clear plaques visible to the naked eye. In contrast, TCID50 assay was rather straightforward. The CPE was very obvious after 7 days of infection and the number of wells displaying CPE was easy to count.

5.3.7 Traditional vs. fast qPCR

The standard curves of the traditional and the fast PCR were presented in Fig 5.5.

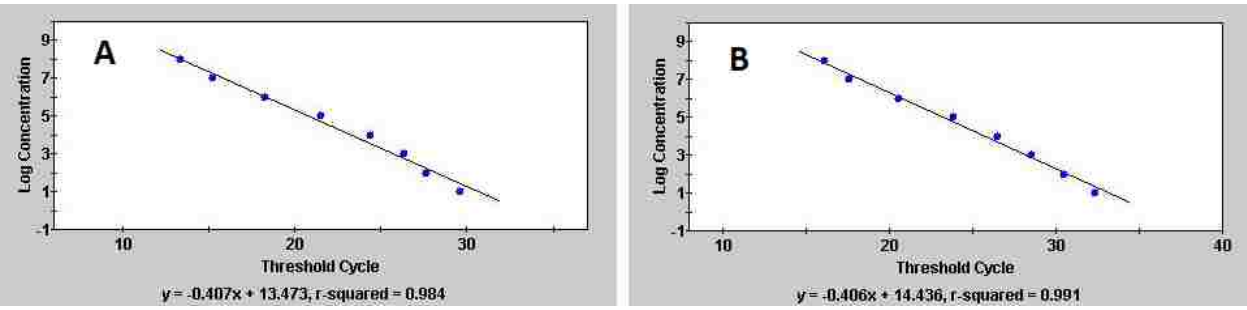

Fig. 5.5 Standard curves of (A) traditional qPCR. (B) fast qPCR

As seen above, the traditional PCR had a detection limit of 0.5-1 log lower than the fast PCR, while the R^2 value of the fast PCR was slightly higher than the traditional PCR. Considering that the traditional PCR took more than 100 min and the fast PCR took less than 30 min, fast PCR would be a less time-consuming method and can be used for rapid detection.

5.4 Discussion

The propagation, cell infectivity assay, and qPCR of HAV were optimized. Our study showed that HAV strain HM175/18f propagated fast in rhesus monkey kidney cells (FRhK-4) and produced obvious CPE in less than one week, while the original host cell line B-SC-1 proved to be not suitable for CPE production. Therefore, FRhK-4 cell line was selected for virus propagation and infectivity assays.

The proper maintenance of cell viability is of extremely importance to the adequate production of infectious virus particles and the reliability of cell infectivity assays. Reviving cryopreserved cells is a critical step in cell culture and subsequent virus propagation, and current

protocols suggested centrifugation and media dilution to diminish the cytotoxicity of DMSO contained in the cryoprotectant. Although centrifugation removes residual DMSO more thoroughly than media dilution, the physical stress also causes cell death. Thus we suggest media dilution as a more reliable method for cell recovery.

Cell culture maintained in 5% CO_2 atmosphere was used widely by studies on HAV (Biziagos et al., 1988; Lemon et al., 1983; Provost and Hilleman, 1979; Romalde et al., 1994; Scholz et al., 1989). The combination of CO_2 in the atmosphere and sodium bicarbonate in the culture media form a buffering system for balanced pH in cell environment. Most cell culture media include phenol red as a pH indicator so that the pH status of the medium is constantly indicated by the color. Usually the culture medium should be changed/replenished if the color turns yellow (acid) or purple (alkali) (GIBCO® Cell Culture Basics Handbook; Sigma-Aldrich Cell Culture Manual). Due to the inconvenience we encountered maintaining a proper CO_2 pressure, we tested the cell growth in a CO_2-free environment with bicarbonate-free cell culture media. The media we used contained phenol red indicator, and through constant monitoring of the cell growth we discovered that even without the carbonate/CO_2 buffering system, the pH of the media never became alkaline, and cell death (indicated by rounded and floated cells) occurrence was scarce. In addition, cells grew fast in CO_2-free environment, requiring only two days to double. We hereby suggest that FRhK-4 cells could be grown in a CO_2-free environment with bicarbonate-free cell culture media. Our study also confirmed that the presence of serum during initial virus inoculation inhibited the propagation of HAV.

Although the majority of studies employ plaque assay to enumerate HAV (Blaise-Boisseaua et al., 2010; Dubois et al., 2007; Kingsley and Richard, 2001), we found that it was very difficult to observe clear plaques, despite repeated efforts in adjusting the plaque assay conditions.

The use of neither agarose nor carboxymethylcellulose succeeded in visible plaque formation, and the supplementation of crystal violet and neutral red for visual aid also failed to increase the visibility of the plaques. Interestingly, even though no plaques were visible to the naked eye, the CPE was very profound under the microscope. Therefore, we applied the TCID50 assay for quantification of HAV. The TCID50 assay is based on the percentage of cells that display CPE compared to the entire cell population that has been infected with serial virus dilutions, and calculation of TCID50 has been well established (Barrett et al., 1996; Parker and Parrish, 1997; Saganuwan, 2011; Schmidt and Emmons, 1989). Based on our experience, the TCID50 assay was simpler and less labor-intensive than plaque assay, and its outcome was far more conclusive and consistent. We conclude that TCID50 assay is a reliable quantification method of HAV.

The sensitivity and accuracy of a fast qPCR procedure developed by Life Technologies was tested. To our knowledge, this was the first report on the validation of a fast qPCR. It was demonstrated that although the fast qPCR was slightly less sensitive than a traditional qPCR, it was accurate and much more time-effective. We conclude that fast qPCR is a reliable quantification method of HAV and is particularly suitable for rapid virus detection.

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Part II: Adsorption of HAV and its indicator from artificial seawater using zeolite

5.6 Introduction

Zeolites are known as molecular sieves, and are widely used in industry for water purification due to low cost, strong ion-exchange abilities and large adsorption capacity (Widiastuti et al., 2008). Zeolites are hydrated crystalline tectoaluminosilicate with uniform pore sizes, and can recognize and organize molecules similar to their pore size (Davis and Lobo, 1992). Some zeolites contain microporous hydrated aluminosilicates crystals with well-defined structures containing AlO_4 and SiO_4 tetrahedral linked through the common oxygen atoms and have a strong affinity for ammonia (Marijaa, et al., 2009). Various studies suggested the ability of zeolite to adsorb viruses and remove contaminants from water. Zeolite was documented to remove 99% of viruses and 100% of *E. coli* from the water, and adsorb up to 5 logs of viruses in less than 1 minute (Abbaszadegan et al., 2006; Bright et al., 2009; Imai et al., 2012; Schulze-Makuch et al., 2002; Schulze-Makuch et al., 2003). Due to the high adsorption capacity of zeolite, it can be used to concentrate suspended virus particles from seawater, and a small volume (1 ml) of eluent can be used to elute adsorbed virus. As no previous studies have conducted their experiment in marine water, this report investigates the efficiency of zeolite to concentrate HAV and its indicator MS2 from artificial seawater.

5.7 Materials and methods

5.7.1 Virus and bacteriophage

Hepatitis A virus (HAV) cytopathic strain HM175/18f (ATCC VR-1402) was grown in FRhK-4 cells (ATCC CRL-1688) for 7 days. Viruses were harvested from infected cell lysates by freeze-thawing. Bacteriophage MS2 (ATCC 15597-B1) was propagated in the exponentially

growing *Escherichia coli* (ATCC 15597) culture for 16 h at 37°C. The overnight culture of MS2 was clarified by centrifugation at 2300 × *g* for 10 min and filtration through a 0.45 µm filter.

5.7.2 Plaque assay

The titer of the MS2 culture was determined by plaque assay. Briefly, 10 ml of bottom agar (1.2%) was poured in 100×15 mm Petri dishes and 8 ml agar (0.6%) containing *E. coli* 6 h culture was poured on top. Supplements of glucose, $CaCl_2$ and thiamine were added to both layers at final concentrations of 0.1%, 2 mM and 10 µg/ml. Thirty microliters of MS2 dilutions were then spread on top of the agar with a hockey stick (bent glass rod). All plates were incubated at 37°C overnight before examined for plaques.

5.7.3 TCID50 assay

The tissue culture infectious dose 50% (TCID50) method was used to determine the titer of HAV stock and dilutions. Healthy FRhK-4 cells were seeded in 6-well tissue culture dishes (Sarstedt Inc., Newton, NC) at 90% confluency and incubated at 37°C. After 24 h of incubation at 37°C, 70 µl of serial diluted viruses were added to each well, with 10 replicates per dilution. The dishes were incubated for 7 days at 37°C and checked daily for characteristic CPE. The TCID50 was calculated using a modified Reed-Muench equation according to Saganuwan (2011).

5.7.4 Extraction of viral RNA

One hundred forty microliters of virus suspension was incubated with 560 µl of lysis buffer (QIAamp Viral RNA mini kit) for 10 min at room temperature. Absolute ethyl alcohol (560 µl) was added to the lysate and mixed by vortexing. The mixture was transferred to a spin column in 630 µl aliquots. The column was washed twice, and the RNA was eluted with 30 µl of RNase-free water containing 0.04% sodium azide and used in PCR immediately.

5.7.5 Real-time PCR (qPCR)

PCR was performed with a SmartCycler (Cepheid, Sunnyvale, CA). For MS2, 10.8 µl of RNA was added to 14.2 µl of mixture containing 0.2 µl 125×RT Enzyme Mix (ArrayScript^{TM} UP Reverse Transcriptase, RNase inhibitor), 12.5 µl 2×RT-PCR Mix (SYBR® Green I dye, AmpliTaq Gold® DNA Polymerase, Ultra-pure, dNTPs, ROX^{TM} passive reference, Optimized buffer components), 0.2 mM of each primer, 10 U RNase inhibitor (Life Technologies, Carlsbad, CA). The thermocycling conditions were (O'Connell et al., 2006): 48°C for 30 min, 95°C for 10 min, 40 cycles of (95°C for 15 s, 60°C for 1 min), then 95°C for 1 min, and 65°C for 2 min. The primers were as follows: forward: 5' GTC GCG GTA ATT GGC GC 3'; reverse: 5' GGC CAC GTG TTT TGA TCG A 3'.

For HAV, 9.3 µl of RNA was added to 10.7 µl of mixture containing 5 µl of TaqMan® Fast Virus 1-Step Master Mix (Life Technologies Co. Carlsbad, CA), 900 µM of each primer, and 150 µM of probe. The thermocycling conditions were (Protocol: TaqMan® Fast Virus 1-Step Master Mix): reverse transcription at 50°C for 5 min, initial denaturation at 95°C for 20 s, and 40 cycles of amplification (95°C for 3 s, 60°C for 30 s). The primers and probe sequences were as follows (Costa-Mattioli et al., 2002): Forward: 5' TTT CCG GAG CCC CTC TTG 3'; reverse 1: 5' AAA GGG AAA TTT AGC CTA TAG CC 3'; reverse 2: 5' AAA GGG AAA ATT TAG CCT ATA GCC 3'; probe: Fam-ACT TGA TAC CTC ACC GCC GTT TGC CT-Tamra.

5.7.6 Artificial seawater

Artificial seawater was made with SeaChem Marine Salt (SeaChem Laboratories Inc. Madison, GA) and distilled water. The salinity of the seawater was adjusted to 10 parts per thousand (ppt) with a salinity meter (YSI Inc., Yellow Springs, OH).

5.7.7 Zeolite

Granular zeolite (Zeobrite®Xtreme) (kindly provided by Mr. Charles Admire from Zeotech Co., Fortworth, TX) was used in this study. The zeolite granules had a size range of 14 × 30 mesh (0.3-1.4 mm dia). Zeobrite®Xtreme was modified by its manufacturer through a chemically bonded carbon chain that made the zeolite "dual-charged". The modified zeolite was reported to have a total surface area of 45-60 m^2/g and can attract positive particles to its interior and negatively charged particles to its surface (Fig 5.6).

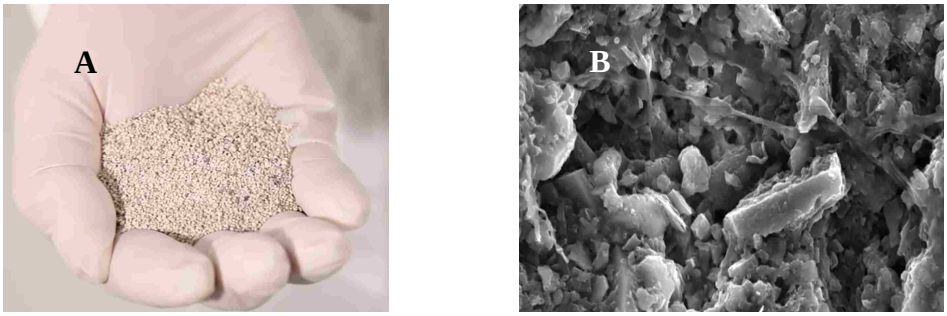

Fig. 5.6 (A) Zeobrite®Xtreme (B) Zeobrite®Xtreme (2000 ×) (www.zeobritextreme.com)

5.7.8 Zeolite concentration of MS2 and comparison with granular activated carbon

Filtration protocol. Our previous study has demonstrated the efficiency of granular activated carbon (Matrix Carbon™) in concentrating bacteriophage MS2 from seawater (Chapter 4). In this study, granular activated carbon was compared to zeolite in their efficiencies to concentrate MS2 from artificial seawater. Briefly, 500 ml of artificial seawater (10 ppt) was spiked with 6~7 log PFU of MS2. The seawater was then filtered by 3 g of zeolite. Thirty microliters of sample was drawn from pre-filtered and post-filtered water and plated in the plaque assay. The same procedure was repeated with granular activated carbon. In addition, 500 ml of seawater was spiked with 6~7 logs of MS2 and filtered by 3 g of granular activated carbon, after which 30 µl of water sample was plated in the plaque assay; the seawater was filtered again by 3 g of zeolite, after which 30 µl of water sample was plated in the plaque assay. Alternatively, 500 ml of spiked

seawater was first filtered by 3 g of zeolite and then by 3 g of granular activated carbon. The quantity of MS2 concentrated by the adsorbent would be reflected in the difference between the pre-filtered and the post-filtered water samples.

Suspension protocol. Five hundred milliliters of seawater was spiked with 6~7 logs of MS2, after which 3 g of activated carbon was suspended in the seawater for 15 min with a magnetic stir bar. The water was then separated from the activated carbon and filtered twice by 3 g of zeolite. Alternatively, 3 g of zeolite was suspended in 500 ml of spiked seawater for 15 min. Thirty microliters of samples were drawn from all pre-filtered and post-filtered water samples and plated in plaque assay.

5.7.9 Concentration and elution of MS2

Five hundred milliliters of seawater (10 ppt) was spiked with 6~7 logs of MS2 and filtered by 3 g of zeolite. One hundred forty microliters of seawater was taken before and after zeolite filtration and used for RNA extraction. One point five milliliters of each of the following eluents: 0.25% trypsin-EDTA solution (Sigma-Aldrich Co. St. Louis, MO), 0.6 M NaCl solution (pH adjusted to 2.6), 0.1% sodium dodecyl sulfate (SDS) and viral RNA lysis buffer (QIAamp Viral RNA mini kit, Qiagen Inc., Valencia, CA) was incubated with the zeolite for 30 min with mild agitation for elution (Cooney et al., 1999a; Cooney et al., 1999b; Zhao et al., 2010). The eluents were separated from the zeolite after 30 min of incubation and briefly centrifuged. RNA was extracted from the supernatant with QIAamp Viral RNA mini kit as described previously and used in PCR immediately.

5.7.10 Concentration and elution of HAV

Fifty milliliters or 500 ml of seawater (10 ppt) were spiked with 6~8 log TCID50 of HAV and filtered by 2 g of zeolite. One hundred forty microliters of seawater was taken before and after

zeolite filtration and used for RNA extraction. One milliliter of eluent was incubated with the zeolite for 30 min with mild agitation for elution. The eluent was separated from the zeolite after 30 min of incubation and briefly centrifuged. RNA was extracted from the supernatant with QIAamp Viral RNA mini kit as described previously and used in PCR immediately.

NaCl solution as eluent. The protein capsid of virus typically contains ionizable amino acids such as glutamic acid, aspartic acid, histidine, and tyrosine (Gerba, 1984). Like many organic chemicals, these individual carboxyl and amino groups, depending on the pH of the surrounding environment, can gain or lose a proton giving the capsid a net electrical charge. Under ambient conditions, most of the enteric viruses are negatively charged (Fong and Lipp, 2005; Sobsey et al., 1973; Sobsey, 1995). Since the zeolite used in this study was modified to have "dual charge" ion exchange property, the ionic strength of the eluent was gradually increased to find the optimal salt concentration at which point the viruses would be eluted. Increasing ionic strength has long been recognized as a way to weaken the bond between virus particles and the adsorbent (Pierce et al., 1979; Zhuang and Jin, 2003). This was attributed to the effect of ion shielding, which at higher ionic strength decreased the electrostatic attraction between the viral particles and the surface of the adsorbent and consequently decreased virus sorption. In this study, NaCl solutions (0.6 M, 1 M, 1.4 M, 1.8 M, 2.2 M, 2.6 M, 3 M, 3.4 M, 4.2 M, 4.6 M, 5 M, 5.4 M and 5.8 M) were used in HAV elution. Since NaCl was the only solute in the eluent, the ionic strength of the eluent depended only on the concentration of the NaCl solution.

Phosphate buffer as eluent. Phosphate buffer has also been documented to be used as an eluent, due to its ability to enhance ionic strength of the solution (Chu et al., 2000; Storkus et al., 1993; Zhuang and Jin, 2003). Chu and others (2000) discovered that MS2 was removed from sand columns in high ionic strength phosphate buffer. The most commonly used phosphate buffers

consist of a mixture of monobasic dihydrogen phosphate and dibasic monohydrogen phosphate. By varying the amount of each salt, a range of buffers can be prepared. Phosphates have a very high buffering capacity and are highly soluble in water (DeAngelis, 2007). Monobasic dihydrogen phosphate reacts with strong base (NaOH) and produces dibasic monohydrogen phosphate; while dibasic monohydrogen phosphate reacts with strong acid (HCl) and produces monobasic dihydrogen phosphate. Thus the amount of monobasic dihydrogen phosphate and dibasic monohydrogen phosphate can be adjusted by adding strong acid or strong base to the solution. In this study, phosphate buffers (pH 1-8) were made from 0.5 M (saturation point) monobasic dihydrogen phosphate and the pH was adjusted with 4 N HCl, while phosphate buffers (pH 9-13) were made from 0.5 M (saturation point) dibasic monohydrogen phosphate and adjusted with 6 N NaOH to the designated pH.

Glycine as eluent. Glycine was recommended as the standard eluent for the recovery of enteric viruses from water, wastewater, and coastal sediments (Gerba et al., 1977a; Gerba et al., 1977b; Gerba et al., 1978; LaBelle and Gerba, 1980; LaBelle et al., 1980; LaBelle and Gerba, 1982; Tsai et al., 1983). In this study, glycine solutions (0.1 M, pH 2; 0.1 M, pH 9; 0.1 M glycine with 0.5 M NaCl and 0.05% Tween 80, pH 8) were used in HAV elution.

Beef extract as eluent. As an alkaline proteinaceous substance, beef extract has been used for recovery of enteric viruses from water and was documented to achieve high recovery rate of virus elution from glass cartridge fiber (Berg et al., 1971; Katzenelson et al., 1976; Landry et al., 1978; Wellings et al., 1975; Wellings et al., 1976). In this study, beef extract (3%, pH 9; 3%, with 10% FBS; 3%, in pH 8 Mcllvaine buffer; 10%, with 10% FBS; 10%, in pH 8 Mcllvaine buffer) were used in HAV elution. The pH of 10% beef extract was further adjusted to a range of 2 to 12 with HCl and NaOH.

Protein denaturant as eluent. In the previous study, trypsin solution was shown to be effective in eluting MS2 from activated carbon. It was concluded that the viral RNA was successfully released due to the disruption of the viral capsid protein by trypsin. In this study, pepsin (0.25% and 10%), proteinase K (200 µg/ml), trypsin-EDTA (0.25%), and urea (8 M) were used in HAV elution.

EDTA as eluent. EDTA (ethylenediaminetetraacetic acid) is an organic chelating agent and used widely to sequester metal ions such as Ca^{2+} and Al^{3+}. Once bound to EDTA, these metal centers tend not to form precipitates or to interfere with the action of other substrates in the environment (Bartlett and Riego, 1972; Bisque, 1961). EDTA has a high affinity for aluminum and was documented to remove aluminum atoms from zeolite (Beaumont and Barthomeuf, 1972). EDTA disodium (0.1%, pH 13; 0.1 M, pH 10; 1%, pH 13; 10%, pH 10) was used in this study due to its higher solubility than EDTA. Since EDTA can inhibit the activity of Mg^{2+}, a cofactor of DNA polymerase that plays a critical role in PCR, $MgCl_2$ solution (1 M) was used in the final step of RNA extraction instead of RNase-free water. The excess Mg^{2+} would "occupy" residual EDTA molecules and prevent them from sequestering the DNA polymerase cofactor.

Surfactant as eluent. Nonionic surfactants Triton X-100 and Tween 80 and ionic surfactant sodium dodecyl sulfate (SDS) are commonly used in virus adsorption/elution to control the surface tension of the fluid and to prevent virus adsorption. While Triton X-100 would be expected to affect nonionic adsorption only, the anionic SDS would be expected to affect both ionic and nonionic adsorption and specifically counteract the adsorption forces (American Society for Testing and Materials, 1995; Fujito and Lytle, 1996; Lytle et al., 1992; Retta et al., 1991). Triton X-100 (5% and 10%), Tween 80 (5% and 10%), and SDS (1%, 2%, 5%, and 10%) were used in HAV elution. For SDS eluates, NaCl was added to the viral lysis buffer to a 0.2 M final

concentration during RNA extraction, since NaCl keeps SDS soluble in 70% ethanol so it won't precipitate with the RNA (Oswald, 2007).

5.8 Results

5.8.1 Zeolite concentration of MS2 and comparison with granular activated carbon

Filtration protocol. The level of MS2 in the artificial seawater filtered by zeolite dropped by 90%. In contrast, the level of MS2 in the water filtered by activated carbon remained the same. Furthermore, seawater filtered first with activated carbon and then with zeolite did not have significantly less MS2 than the water filtered with only zeolite. Interestingly, water filtered first with zeolite and then with activated carbon had even more MS2 remained.

Suspension protocol. Suspending granular activated carbon in the artificial seawater reduced the level of MS2 by 60%, and further filtration with zeolite dropped the MS2 level by an additional 15~20%. Suspending zeolite in the seawater reduced the level of MS2 by 90%.

5.8.2 Concentration and elution of MS2

Interestingly, PCR amplification of the spiked seawater sample filtered by zeolite revealed that only ~60% MS2 was removed from the water, unlike the results from the plaque assay. Furthermore, trypsin-EDTA, SDS and viral lysis buffer all failed in eluting MS2 from zeolite, since no positive PCR amplification was observed from eluates. Only 0.6 M NaCl had moderate success; 1.5 ml of NaCl solution eluted 3.7 out of 7.3 log PFU of MS2 concentrated by zeolite.

5.8.3 Concentration and elution of HAV

PCR amplification of the spiked seawater sample filtered by zeolite revealed that ~90% HAV was removed from the water. The removal rate fluctuated from 90% to 99% in 50 ml seawater, and from 80% to 90% in 500 ml seawater. It was concluded that the removal rate depended on the flow rate of the seawater; higher flow rate meant heavier stream and less contact

between zeolite and the suspended virus particles. Since an automated system was not available, the flow rate was controlled manually.

Surprisingly, the majority of eluents tested showed inability to elute HAV from zeolite, as indicated by negative PCR amplification of the eluates. Ten percent beef extract (maintained in pH 8 Mcllvaine buffer) and SDS (2%, 5%, 10%) were the only eluents able to elute HAV from zeolite, but SDS far exceeded beef extract regarding elution efficiency. Beef extract (10% (v/v) in pH 8 Mcllvaine buffer) was only able to elute 3.7 out of 7.8 logs of adsorbed HAV from zeolite, while 5% SDS was able to elute 5.3 out of 7.8 logs of HAV.

5.9 Discussion

Consistent with the observations in the previous study, granular activated carbon was able to remove a significant amount of MS2 from spiked seawater through 15 min of suspension. However, simply filtering the seawater through activated carbon did not result in any removal of MS2. On the other hand, zeolite was able to remove 90% of MS2 from the water through filtration, which was 30% more than what granular activated carbon removed by suspension. Besides, pre-treating seawater with activated carbon through either filtration or suspension before filtering with zeolite was slightly less effective than filtering the water with only zeolite. Surprisingly, plaque assay and qPCR told two different stories; plaque assay indicated a 90% removal rate by zeolite, while qPCR indicated only 60% removal rate. The difference in results may be due to the sensitivity and specificity of PCR, or inhibition of plaques in marine samples.

Cell infectivity assay wasn't used in the concentration/elution of HAV due to the time-consuming nature of HAV propagation *in vitro*. Instead, qPCR was used as an indication of how efficient zeolite was in virus concentration. Our study suggested that zeolite was able to remove up

to 99% of HAV from the seawater. It appeared that zeolite was much more efficient at concentrating HAV than MS2.

While NaCl solution had moderate success in eluting MS2, it failed to release bound HAV from zeolite. A variety of other commonly used eluents were also unsuccessful at the task. It was concluded that MS2 was adsorbed by zeolite via electrostatic attraction, but it was unclear the types of interaction involved in the adsorption of HAV. The basic building blocks of zeolite form a network of pores in the crystal, and the pores have dimensions that are comparable with the size of the molecules that can be adsorbed (Smit and Maesen, 2008). In addition, the zeolite used in this study have modified surface and can be used as an amphoteric ion-exchanger. The interaction between zeolite and HAV is likely to be surface-based adhesion, and could be either physisorption or chemisorption. The fundamental interacting force of physisorption is caused by van der Waals force, thus the adsorption is non-specific and reversible and can occur at low temperature. Chemisorption on the other hand involves a chemical reaction between the surface and the adsorbate, and is thus highly specific and relatively irreversible. Ion-exchange is a type of chemisorption (Oura et al., 2003). In this study, zeolite easily removed 99% of the suspended HAV from the seawater at room temperature, thus the attraction between zeolite and HAV is more likely to be a form of chemisorption. Due to the ion-exchange property of zeolite and weak negative charge of HAV, we propose to consider the concentration via a protein ion-exchange model. Established protein ion-exchange models suggest that the adsorption of protein onto an adsorbent is primarily electrostatic attraction, but also involve interplay of colloidal van der Waals, short-range repulsion forces, short-range attraction, surface charge density, and size dependent interaction (Roth et al., 1996).

We also attempted to use chelation to release HAV, to no avail. EDTA is commonly used as a chelating agent and can diminish the activity of metal ions and has high affinity towards calcium and aluminum. Since aluminum is responsible for the ion-exchange property of zeolite, it was expected that EDTA would sequester aluminum and reverse any electrostatic attraction formed by zeolite. Since it was observed that using EDTA as an eluent was ineffective at eluting HAV, we further concluded that electrostatic attraction wasn't the sole contribution to the adsorption of HAV.

We theorized that the pore size of the zeolite used in this study was very close to the size of HAV capsid protein, thus the virus particles that were adsorbed onto zeolite remained in the pores and since adsorption occurs via short-distance interaction, common eluents could not access the pores that were occupied by the viruses. The sole success of SDS suggested that a protein denaturant was necessary in releasing HAV from the pores, possibly by unfolding the capsid protein and releasing the viral RNA. The failure of protease to release adsorbed HAV may be due to the size of the protease too large to access the pores.

Zeolite was efficient in concentrating HAV and its indicator MS2 in the tests. It was suggested that MS2 was adsorbed by zeolite via electrostatic attraction, and bond MS2 could be eluted by increasing the salt concentration in the eluent; while concentration of HAV likely involved a more complex mechanism and ionic surfactant SDS was required to elute HAV from zeolite. A concentration/elution method was developed accordingly and optimized for virus concentration in seawater.

5.10 References

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Part III: Concentration and detection of HAV from artificial seawater using zeolite

5.11 Introduction

Current concentration methods can be time-consuming, expensive, and require additional concentration steps. To seek an alternate concentration method with reduced time and cost, the ability of zeolite to concentrate HAV from seawater was assessed. Natural zeolite is a porous aluminum silicate with Si–O and Al–O bond tetrahedron as building unit (Jacobs. 1977). Because the Si^{4+} could be replaced by Al^{3+}, the tetrahedron structure is negatively charged and cations could be adsorbed into the tetrahedron (Niu and Feng, 2005). As a result, zeolite not only possesses great surface area and water adsorption ability but also exhibits strong ion-exchange properties and thus is very suitable for virus extraction. The zeolite used in this study was made from a type of zeolite called clinoptilolite. Due to its negative surface charge, clinoptilolite is known as an ion-exchange mineral for its ability to attract and bond certain positive charged ions. however, since the zeolite was subjected to a patent-pending process by its manufacturer to alter the surface charge from negative to positive, this process maintains the internal negative charge of the zeolite crystals, allowing the zeolite particles to become a dual charged material and remove both negative and positive charged particles that are suspended in water. Electrophoretic studies of viruses indicate that most viruses are negatively charged near neutral pH and have isoelectric points below pH 7 (Hou et al., 1980; Sobsey and Jones, 1979). HAV is a non-enveloped virus enclosed in a spherical capsid protein and the genome-linked protein of HAV was reported to have an isoelectric point of 7.15 (Weitz et al., 1986), and the pH of natural seawater is usually 7.4-8.4 (Roekens and Grieken, 1983). Due to the dual charged property of this zeolite material, HAV could be extracted from seawater without acidification of the water.

In the preliminary study, zeolite was able to adsorb 99% and up to 7.8 log TCID50 of HAV from seawater. This level of concentration efficiency was achieved by merely passing seawater through zeolite without repetition, and is expected to near 100% if the contact between seawater and zeolite is maximized by mechanisms such as vortex. As no previous studies have used zeolite for virus concentration from seawater, this report investigates the efficiency of large-scale (ten liters) concentration of HAV from seawater using zeolite.

5.12 Materials and methods

5.12.1 Host cell line and virus strain

HAV strain HM175/18f (ATCC VR-1402) was propagated in fetal rhesus monkey kidney (FRhK-4) cells (ATCC CRL-1688). The cell culture media contained 20% (w/w) fetal bovine serum (Lonza, Walkersville, MD) in L-15 medium (Sigma-Aldrich Co. St. Louis, MO). The cells were maintained in 37°C incubator with 95% humidity.

5.12.2 Cell passage

Cells were passaged when the confluency reached 90%. Trypsin-EDTA (0.25%) (Sigma-Aldrich Co. St. Louis, MO) was used to aid the passaging. Briefly, the cell culture media was discarded and the cells were rinsed once with 3 ml of trypsin-EDTA before 2 ml of trypsin-EDTA was added to the flask. The flask was incubated at 37°C for 5~10 min to aid the detachment of cells. Ten milliliters of fresh cell culture media was added to the flask and gently pipetted up and down to flush the cells off and to form a homogenous cell suspension. More cell culture media was added to the flask and gently mixed. The volume of cell culture media depended on how many flasks the cells were split into; on average one T75 flask would contain 15 ml of cell culture media. The homogenized cell suspension was evenly distributed to the new flasks and each flask was incubated at 37°C.

5.12.3 Cell preservation

Confluent cells (90%) were detached with the aid of 2 ml of trypsin-EDTA and 10 ml of cell culture media and centrifuged at 1000 rpm (200 × *g*) for 10 min. The supernatant was discarded and the cell pellet was re-suspended in 2 ml of cryopreservation media containing 20% serum and 5% Dimethyl sulfoxide (DMSO) and immediately transferred to two cryogenic vials and preserved at -80°C.

5.12.4 HAV propagation

FRhK-4 cells were ready for infection when the confluency reached 80%~90%. The cell culture media was discarded and 5 ml of serum-free L-15 medium was used to rinse the cells. Seven point five milliliters of serum-free L-15 medium was then added to the cells. Frozen HAV was thawed at room temperature and 0.5 ml of the virus stock was inoculated into the cell culture. The cells were incubated at 37°C for 2 h with constant rocking, after which fetal bovine serum was added to the cell culture to 2%. The infected cells were incubated for 7~10 days, until obvious CPE were seen. The tissue culture flask was then frozen at -80°C for at least one day and thawed at room temperature to disrupt the cells and release the HAV. The virus was aliquoted and preserved at -80°C.

5.12.5 Viral titration

The tissue culture infectious dose 50% (TCID50) method was used to determine the titer of HAV stock and dilutions. Healthy FRhK-4 cells were seeded in eight 6-well plates (Sarstedt Inc., Newton, NC) at 90% confluency and incubated at 37°C for 24 h. Serial 10 fold dilutions of HAV were made in serum-free L-15 medium. The 6-well tissue culture dishes were rinsed with L-15 medium, and 1 ml of serum-free L-15 medium and 70 µl of each serial dilution was then added to each of ten wells of two 6-well dishes. One well of each dish was used as negative

control in which no virus dilution was inoculated. Four serial dilutions were used; each with a 10-fold difference in dilution factor. Thus in total eight 6-well dishes were used in one TCID50 assay. The plates were incubated at 37°C for 7~10 days, until obvious CPE were seen. The TCID50 was calculated according to Saganuwan using a modified Reed–Muench equation (Saganuwan, 2011).

5.12.6 qPCR

PCR was performed in a total volume of 20 µl with a SmartCycler (Cepheid, Sunnyvale, CA). In each reaction, 9.3 µl of RNA was added to 10.7 µl of mixture containing 5 µl of TaqMan® Fast Virus 1-Step Master Mix (Life Technologies Co. Carlsbad, CA), 900 µM of each primer, and 150 µM of probe. The thermocycling conditions were (Protocol: TaqMan® Fast Virus 1-Step Master Mix): reverse transcription at 50°C for 5 min, initial denaturation at 95°C for 20 s, and 40 cycles of amplification (95°C for 3 s, 60°C for 30 s). The primers and probe sequences were as follows (Costa-Mattioli et al., 2002): Forward: 5' TTT CCG GAG CCC CTC TTG 3'; reverse 1: 5' AAA GGG AAA TTT AGC CTA TAG CC 3'; reverse 2: 5' AAA GGG AAA ATT TAG CCT ATA GCC 3'; probe: Fam-ACT TGA TAC CTC ACC GCC GTT TGC CT-Tamra.

5.12.7 Standard curve

Standard curve was performed by testing 10-fold dilutions of HAV. The RNA was extracted from 10^{-1}-10^{-8} HAV dilutions using QIAamp Viral RNA mini Kit (Qiagen Inc., Valencia, CA). The PCR standard curve was constructed from quantifying all the RNA samples in one reaction and the TCID50 of each sample was correlated to the cycle threshold (Ct) value (Butot et al., 2007; Di Pasqualea et al., 2010; Houde et al., 2007). The sensitivity of the assay

was expressed as the lowest number of TCID50 which could be detected. The amplification efficiency (E) was calculated using the following equation: $E = (10^{-slope}) - 1$ (Klein et al., 1999).

5.12.8 Concentration and elution

Artificial seawater of salinities 10, 20, 30 and 40 ppt were made from SeaChem Marine Salt (SeaChem Laboratories Inc. Madison, GA) and inoculated with 6.4~7.8 log TCID50 of HAV. Each step of the concentration and elution methods is shown in Fig 5.7.

Fifty milliliters to two liters of spiked seawater was passed through two grams of zeolite for virus concentration (Zeobrite®Xtreme, Zeotech Co., Fortworth, TX). One milliliter of sodium dodecyl sulfate (SDS) was used for elution.

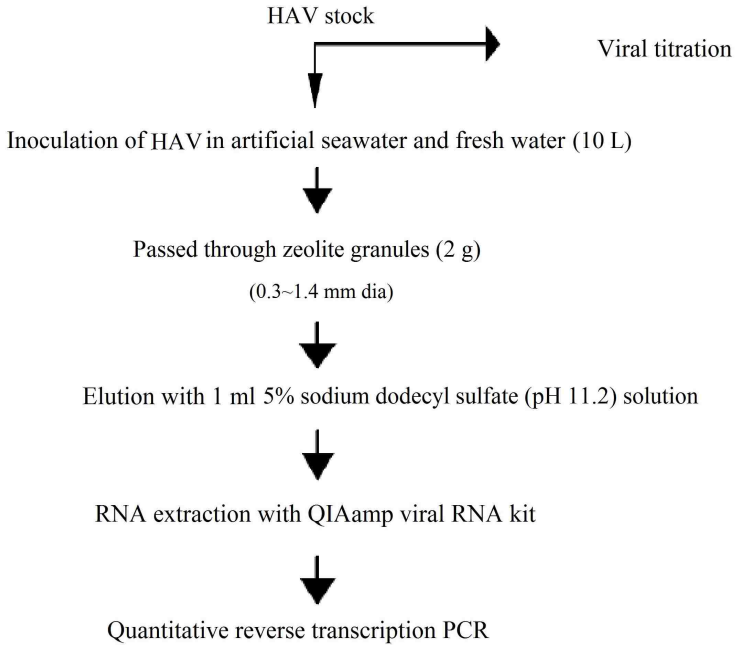

Fig. 5.7 Protocol for concentration of HAV from inoculated artificial seawater

5.12.9 Concentration efficiency

To test the efficiency of virus concentration by zeolite, RNA was extracted from 140 µl pre-filtered water sample and 140 µl post-filtered water sample and qPCR was performed. The amount of HAV detected from post-filtered seawater was then compared to that from the pre-filtered seawater and the concentration efficiency was calculated using the following equation:

$E = 1- (\text{Quantity of HAV after zeolite filtration}/\text{Quantity of HAV before zeolite filtration})$.

5.12.10 Extraction of viral RNA from eluate

The eluate was separated from the zeolite after 30 min of incubation and briefly centrifuged. The supernatant was used for RNA extraction with QIAamp Viral RNA mini kit. Every 140 µl of the supernatant was incubated with 560 µl of lysis buffer (QIAamp Viral RNA mini kit) for 10 min at room temperature. Forty-five microliters of 5.8 M NaCl and 560 µl absolute ethyl alcohol were added to the lysate and mixed by vortexing. NaCl was added to the viral lysis buffer to a 0.2 M final concentration to keep SDS soluble in ethanol so it won't precipitate with the RNA (Oswald, 2007). The mixture was transferred to a spin column in 630 µl aliquots. The column was washed twice, and the RNA was eluted with 30 µl of RNase-free water containing 0.04% sodium azide and used for PCR immediately.

5.12.11 Optimization of SDS

The concentration and pH of SDS were optimized for maximum elution efficiency. Concentrations (1%, 2%, 5% and 10%) and pH (1-13) (maintained by 0.5 M phosphate buffer) of SDS were adjusted and tested for virus elution.

5.12.12 Optimization of elution temperature and time span

The temperature and time span of virus elution were optimized for maximum elution efficiency. Zeolite was incubated with 1 ml of SDS for 30 min at 30°C, 42°C and 100°C, and the

optimal temperature was selected based on the quantity of eluted HAV. Virus elution was then performed at 15 min, 30 min, 60 min and 120 min, for selection of the optimal time span.

5.12.13 Effect of salinity on concentration efficiency

The impact of salinity on the efficiency of the concentration method was tested by inoculating the same level of HAV (7.7 logs per 50 ml water) into artificial seawater of different salinities (0, 10, 20, 30 and 40 ppt) and comparing the quantity of eluted HAV under each salinity.

5.12.14 Large scale concentration of HAV

Seven logs of HAV were inoculated into 10 L of artificial seawater (10 ppt) and 140 µl of sample was taken. Two grams of zeolite was suspended in the artificial seawater by manual stirring and the concentrated HAV was eluted from zeolite with the optimal eluent under the optimal elution temperature and time span. RNA was extracted from the water sample and the eluate, and quantified by qPCR. The quantity of HAV in the water sample was compared to that in the eluate.

5.12.15 Statistical analysis

The effects of salinity, elution temperature and time span on the concentration/elution efficiency were analyzed statistically by comparing the means of HAV detected under each condition. Pairwise comparisons were carried out with Tukey's HSD by JMP (SAS Institute Inc. Cary, NC).

5.13 Results

5.13.1 Standard curve

A linear relationship was observed between the input RNA and the Ct values with correlation coefficients (R^2) of 0.994 and amplification efficiency of 93.64%. The PCR was fast (30 min) and sensitive (detection limit < 20 TCID50) amplification (Fig 5.8).

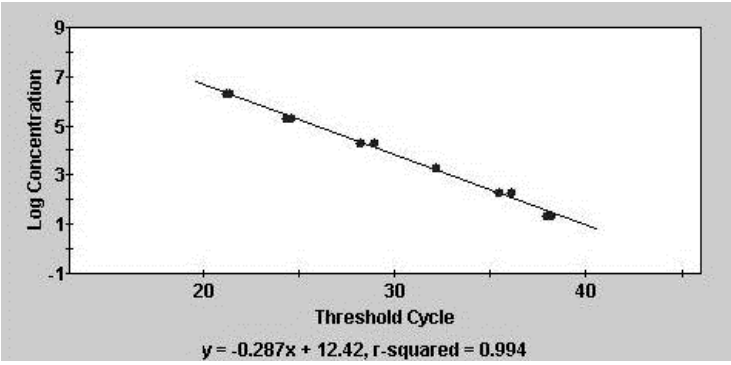

Fig. 5.8 PCR standard curve of HAV

5.13.2 Efficiency of zeolite concentration

PCR detection of spiked water sample before and after zeolite filtration revealed 96.4±3.1% concentration efficiency, regardless of inoculation level (up to 7.8 logs). The efficiency remained constant (99%) when the water volume was scaled up to 500 ml, but dropped when the volume was scaled up to 2 L. It was probably because more water was flushing down in a heavier flow, and some zeolite granules were flushed away and lost contact with the water.

5.13.3 Optimization of SDS

SDS was completely unable to elute HAV at 1% and 2%, and was only effective at 5% and 10%. In addition, not only did 5% SDS eluted 0.7 logs more HAV from zeolite than 10% SDS, it was also far less foamy and thus easier to handle. Furthermore, SDS (5%) dissolved in basic phosphate buffer (pH 8-12) eluted 1-3 logs more HAV than SDS dissolved in acidic buffer (pH 1-7) (Fig 5.9). SDS had the highest elution efficiency in phosphate buffer of pH 11.2 (Fig 5.9).

5.13.4 Optimization of elution time and temperature

Incubating zeolite with 5% SDS (pH 11.2) at 42°C eluted ~0.5 log TCID50 more HAV than at 30°C, and 3 log TCID50 more HAV than at 100°C. Adjustment of elution time revealed optimal elution efficiency at 60 min (Fig 5.10).

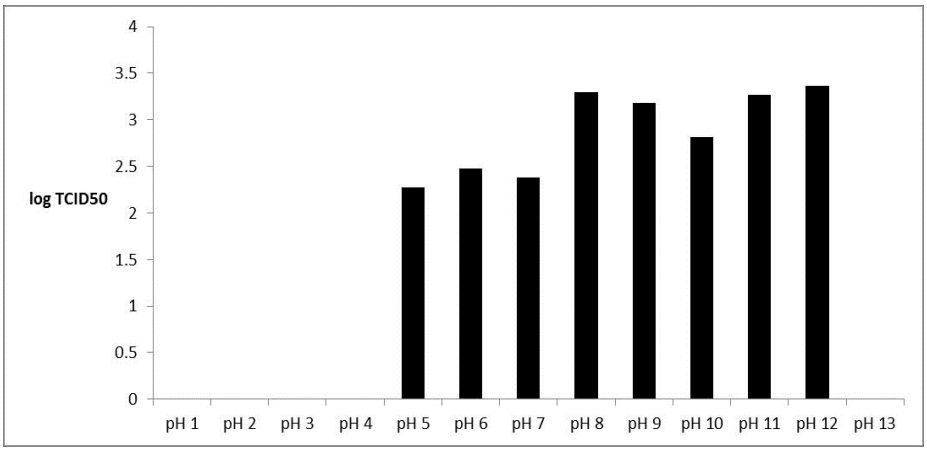

Fig. 5.9 Quantity of HAV concentrated by zeolite and eluted by 5% SDS solution of different pH. Concentration took place in 50 ml of seawater spiked with 5.9 log TCID50 of HAV.

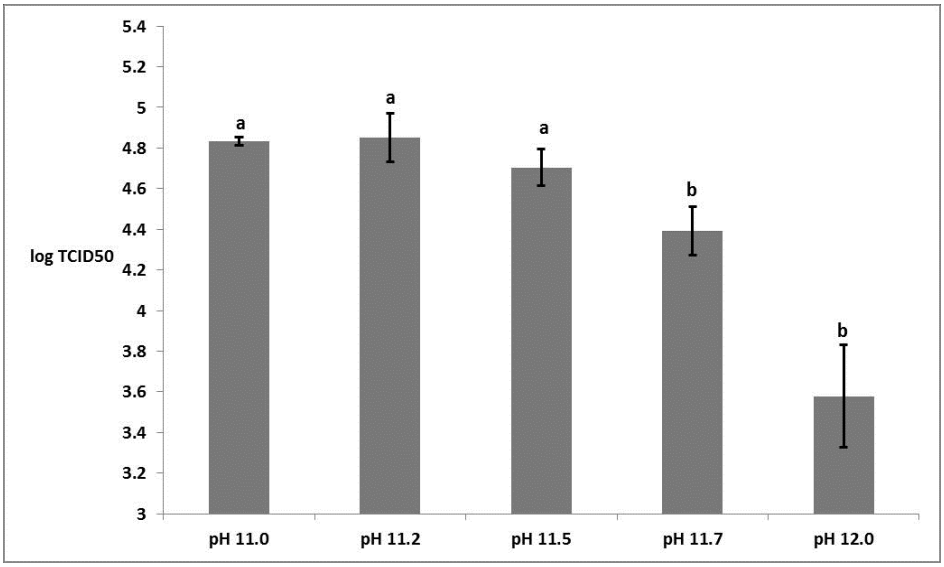

Fig. 5.10 Quantity of HAV concentrated by zeolite and eluted by 5% SDS solutions of pH 11 to 12. Concentration took place in 50 ml of seawater spiked with 7.4 log TCID50 of HAV. The statistical connecting letters were displayed under each condition.

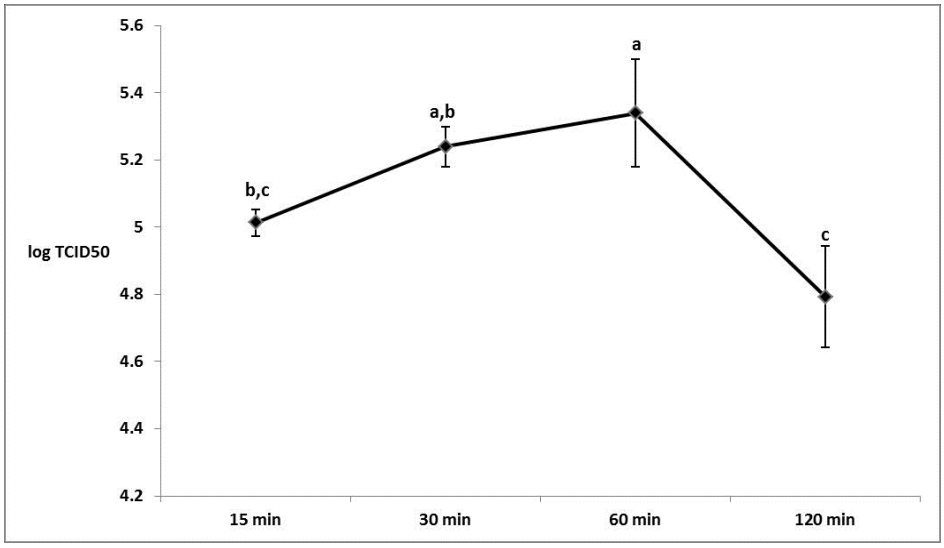

Fig. 5.11 Quantity of HAV concentrated by zeolite and eluted by 5% SDS solution (pH 11.2) under different elution time span. The statistical connecting letters were displayed under each condition.

5.13.5 Effect of salinity on concentration efficiency

The concentration efficiency was much higher in seawater than in fresh water. The efficiency was highest in seawater of 10 ppt, and 5.5 out of 7.8 logs of HAV was detected (Fig 5.12). Even though the efficiency varied in water of different salinities, zeolite concentration was overall effective, being able to detect at least 4.8 out of 7.8 logs of HAV.

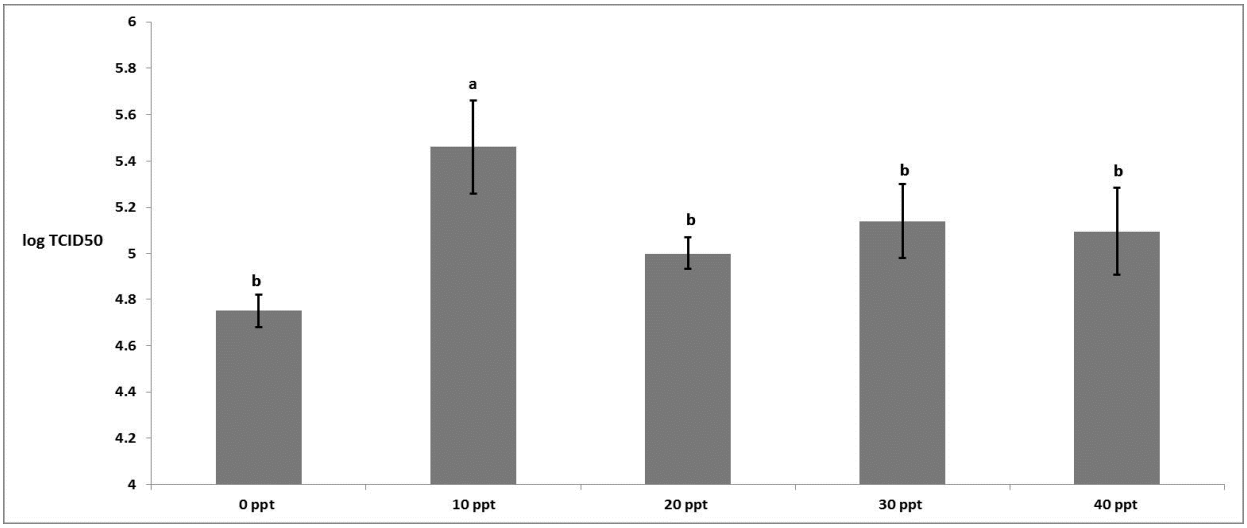

Fig. 5.12 Quantity of HAV detected by zeolite concentration/elution under different salinity conditions. Concentration took place in 50 ml of seawater spiked with 7.8 log TCID50 of HAV.

5.13.6 Large scale concentration of HAV with zeolite

PCR detected 4.0 logs of HAV from 10 L of seawater with zeolite concentration. In comparison, direct extraction of RNA from spiked seawater detected only 2.3 logs. In addition, the entire concentration and detection only took 2 h to complete.

5.14 Discussion

It was observed from repeated trials that the level of detected HAV was approximately 2.3 logs lower than the level of inoculated HAV, regardless of the seawater volume. Since zeolite was able to concentrate 99% of the virus from the seawater, once the seawater volume is scaled up, the concentration/elution method will be very effective and the sensitivity will be even more profound compared to direct detection.

Anions in the seawater such as chloride have much higher ionic strength than HAV and should theoretically prevent the adsorption of HAV. The observation that zeolite concentration was even more efficient in seawater than in fresh water suggested that electrostatic attraction was not the only force involved in virus concentration. This hypothesis was further supported by the fact that sodium chloride failed to elute HAV off zeolite. Theoretically by increasing the salt concentration (salt gradient) in the eluent, the molecules bond to the adsorbent should start to elute. However, in the preliminary study it was observed that by increasing the concentration of sodium chloride to its saturation (5.8 M), HAV was still not detected in the eluate.

Zeolite can effectively concentrate HAV from seawater without the need to modify the acidity or salinity of the seawater, and only a small volume of eluent (1 ml) is needed to elute virus particles from zeolite, with no secondary concentration procedure necessary. Zeolite could potentially be used for virus concentration from shellfish growing waters at low cost, and be conveniently transported to the lab for analysis.

5.15 References

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Chapter 6. Conclusions

Enteric viruses such as hepatitis A virus (HAV) are responsible for a large proportion of food and water-borne illnesses. These viruses are transmitted to humans via the fecal-oral route, usually from contaminated water or foods such as raw shellfish. HAV infection is the leading worldwide cause of acute viral hepatitis, and its outbreaks have occurred among consumers of shellfish harvested from fecal polluted waters. HAV is often detected in shellfish, even when levels of fecal coliforms are low.

Viral contaminated shellfish can be a public health concern, as outbreaks of gastroenteritis have occurred among consumers of shellfish harvested from fecal polluted waters. Shellfish can actively bio-concentrate HAV as much as 100 fold from fecal contaminated waters, and even point source discharge of human waste from commercial and recreational vessels can result in viral contamination of approved shellfish beds without observation of increase in fecal coliform in marine water samples.

A rapid detection of viral contamination in water environments can prevent economic loss and identify the source of contamination within a short time for public safety. Although HAV can be elucidated by cell culture and molecular techniques, they are usually expensive and time-consuming for routine screening. Therefore it's important to find a suitable indicator organism for the routine screening of the presence of HAV. Male-specific (FRNA) bacteriophages have been proposed as indicators of enteric viruses. Bacteriophage assay conditions are much simpler and cheaper than enteric virus detection methods. The abundance of these phages in sewage, and the ease with which they can be enumerated make them attractive indicators of viral contamination in the environment. As a male-specific RNA coliphage that infects *Escherichia coli*, MS2 is used

widely as a surrogate for enteric viruses, and its presence has been proven to be a reliable indication of the presence of HAV.

Numbers of viruses in water are usually too low for detection; however, even one infective HAV is sufficient to cause hepatitis A infection in humans. Therefore, large volumes of water (10-20 L) needs to be concentrated before analysis can be carried out. Different types of filters and filtration methods have been used to collect and concentrate viral particles from water samples; however, most filters are either easily clogged and have low recovery rates for viruses in marine water, or are costly and time-consuming. Concentrated samples usually need to be further concentrated to reduce the final volume of samples to 1 or 2 ml for processing, and procedures of secondary concentration have often been reported to cause inhibitory effects in PCR assays.

We successfully developed a method to concentrate hepatitis A virus and its surrogate bacteriophage MS2 from artificial seawater. Virus particles were successfully concentrated from the seawater by an adsorbent with large internal surface area and/or ion exchange property and eluted with a small volume (1 ml) of protein denaturant. Detection was carried out with qPCR. During the method development, we also optimized the current cell culture, cell infectivity assay and qPCR detection of HAV and its surrogate MS2.

We tested granular activated carbon and zeolite as adsorbents to be used in the virus concentration method and compared their respective efficiencies. While both adsorbents displayed relatively high efficiencies in virus concentration, zeolite was superior to granular activated carbon; not only did it concentrate more viruses from the seawater, but it also took less time to achieve this efficiency. Zeolite also displayed a higher efficiency in concentrating HAV than its indicator MS2.

Zeolite was established to be highly effective in concentrating HAV from artificial seawater and the subsequent elution was relatively efficient. Zeolite can concentrate HAV from

seawater without the need to modify the acidity or salinity of the seawater, and only a small volume of eluent (1 ml) is needed to elute virus particles from zeolite, with no secondary concentration procedure necessary. Zeolite has the potential to be used in virus concentration from shellfish growing waters and be conveniently transported on land. Compared to existing methods, this method doesn't require a secondary concentration step or the need to modify the pH and salinity of the seawater. Furthermore, this method does not involve complex machineary and can be done in approximately 2 h. In conclusion, the virus concentration and detection method using granular adsorbent is sensitive, rapid, simple and inexpensive, and can be applied in rapid virus detection from seawater.

Appendix 1. List of different viruses and host cells used

Species	Strain	Culture number	Origin	Source [a]
Hepatitis A virus	HM175/18f	ATCC VR-1402	Human fecal material passaged in marmosets.	ATCC
Rhesus monkey kidney cells	FRhK-4	ATCC CRL-1688	Female rhesus monkey (*Macaca mulatta*) kidney	ATCC
African green monkey kidney cells	BS-C-1	ATCC CCL-26	African green monkey (*Cercopithecus aethiops*) kidney	ATCC
MS2 bacteriophage	MS2	ATCC 15597-B1	N/A	ATCC
Escherichia coli	C-3000	ATCC 15597	Derived from existing strain	ATCC

[a] ATCC- American Type Culture Collection

Appendix 2. List of media and reagents used

1) *Escherichia coli* medium-Medium base-(Tryptone-10 g, Yeast extract-1 g, NaCl-8 g, d. water-1 L). Boil to dissolve. Supplement 1-Glucose-5 g, d. water-50 ml. Dissolve the ingredient and sterilize through 0.45 µm filter. Supplement 2-Calcium chloride dihydrate-7.35 g, d. water-50 ml. Dissolve the ingredient and sterilize through 0.45 µm filter. Supplement 3-Thiamine-0.5 g, d. water-50 ml. Dissolve the ingredient and sterilize through 0.45 µm filter. Add 10 ml supplement 1, 2 ml supplement 2, and 1 ml supplement 3 to cooled medium base, mix.
2) Plaque assay top layer agar-Agar base-(Tryptone-2 g, Yeast extract-0.2 g, NaCl-1.6 g, Agar-0.9 g, d. water-200 ml). Boil to dissolve. Supplement 1- Glucose-5 g, d. water-50 ml. Dissolve the ingredient and sterilize through 0.45 µm filter. Supplement 2-Calcium chloride dihydrate-7.35 g, d. water-50 ml. Dissolve the ingredient and sterilize through 0.45 µm filter. Supplement 3-Thiamine-0.5 g, d. water-50 ml. Dissolve the ingredient and sterilize through 0.45 µm filter. Add 2 ml supplement 1, 0.4 ml supplement 2, and 0.2 ml supplement 3 to cooled medium base, mix, dispense into petri dishes.
3) Plaque assay bottom layer agar-Agar base- (Tryptone-2 g, Yeast extract-0.2 g, NaCl-1.6 g, Agar-2 g, d. water-200 ml). Boil to dissolve. Supplement 1- Glucose-5 g, d. water-50 ml. Dissolve the ingredient and sterilize through 0.45 µm filter. Supplement 2-Calcium chloride dihydrate-7.35 g, d. water-50 ml. Dissolve the ingredient and sterilize through 0.45 µm filter. Supplement 3-Thiamine-0.5 g, d. water-50 ml. Dissolve the ingredient and sterilize through 0.45 µm filter. Add 2 ml supplement 1, 0.4 ml supplement 2, and 0.2 ml supplement 3 to cooled medium base, mix, dispense into petri dishes.
4) Phosphate Saline buffer (PBS)-(NaCl-7.65g, Na_2HPO_4-0.724 g, KH_2PO_4-0.210 g, d. water- 1 L). Dissolve the ingredients and adjust the pH to 7.

5) Agarose solution-agarose base-(agarose-2.5 g, PBS-50 ml). Autoclave at 121°C for 15 min. Medium base-(L-15 medium-42.5 ml, FBS-2.5 ml). Add 5 ml agarose base to 45 ml medium base, mix, dispense into tissue culture dishes.

6) Carboxymethylcellulose solution-Carboxymethylcellulose base-(carboxymethylcellulose 0.4 g, L-15 medium-15 ml). Dissolve the ingredient and sterilize through 0.45 µm filter. Medium base-L-15 medium-18.8 ml, FBS-1.2 ml. Add 15 ml carboxymethylcellulose base to 20 ml medium base, mix, dispense into tissue culture dishes.

7) NaCl solution-NaCl-27.12 g, d. water-80 ml. Dissolve the ingredient and dispense 10 ml for 5.8 M NaCl. Add 5.2 ml d. water to remaining solution, mix, dispense 10 ml for 5.4 M NaCl. Add 5.2 ml d. water to remaining solution, mix, dispense 10 ml for 5 M NaCl. Add 5.3 ml d. water to remaining solution, mix, dispense 10 ml for 4.6 M NaCl. Add 5.3 ml d. water to remaining solution, mix, dispense 10 ml for 4.2 M NaCl. Add 5.3 ml d. water to remaining solution, mix, dispense 10 ml for 3.8 M NaCl. Add 5.5 ml d. water to remaining solution, mix, dispense 10 ml for 3.4 M NaCl. Add 5.5 ml d. water to remaining solution, mix, dispense 10 ml for 3 M NaCl. Add 5.8 ml d. water to remaining solution, mix, dispense 10 ml for 2.6 M NaCl. Add 6 ml d. water to remaining solution, mix, dispense 10 ml for 2.2 M NaCl. Add 6.5 ml d. water to remaining solution, mix, dispense 10 ml for 1.8 M NaCl. Add 7.3 ml d. water to remaining solution, mix, dispense 10 ml for 1.4 M NaCl. Add 9.1 ml d. water to remaining solution, mix, dispense 10 ml for 1 M NaCl.

8) Phosphate buffer-NaH_2PO_4-30 g, d. water-500 ml. Dissolve the ingredient and dispense every 50 ml. Adjust the pH of each solution to a series of 1 to 8 with 4 N HCl.

9) Phosphate buffer-Na_2HPO_4-17.75 g, d. water-250 ml. Dissolve the ingredient and dispense every 50 ml. Adjust the pH of each solution to a series of 9 to 13 with 6 N NaOH.

10) Glycine solution-Glycine- 0.75 g, d. water-10 ml. Dissolve the ingredient and dispense 0.5 ml into 4.5 ml pH 2 phosphate buffer for 0.1 M glycine (pH 2). Dispense 0.5 ml into 4.5 ml pH 9 phosphate buffer for 0.1 M glycine (pH 9). Dispense 0.5 ml into 2 ml d. water and 2.5 ml 1 M NaCl, add 2.5 µl Tween 80 for 0.1 M glycine, 0.5 M NaCl, 0.05% Tween 80 solution.

11) McIlvaine's buffer-Na_2HPO_4-0.71 g, Citric acid-0.12 g, d. water-100 ml. Dissolve ingredients and adjust the pH to 8.

12) Beef extract-Beef extract powder-0.3 g, d. water-10 ml. Dissolve ingredients and adjust the pH to 9 for 3% beef extract (pH 9). Beef extract powder-0.3 g, fetal bovine serum-1 ml, d. water-9 ml. Dissolve ingredients for 3% beef extract with 10% FBS. Beef extract powder-0.3 g, McIlvaine's buffer-10 ml. Dissolve ingredients for 3% beef extract in Mcllvaine’s buffer. Beef extract powder-1 g, fetal bovine serum-1 ml, d. water-9 ml. Dissolve ingredients for 10% beef extract with 10% FBS. Beef extract powder-1 g, McIlvaine's buffer-10 ml. Dissolve ingredients for 10% beef extract in Mcllvaine’s buffer.

13) EDTA solution-EDTA disodium-0.372 g, d. water-10 ml. Dissolve ingredients and adjust the pH to 10 for 0.1 M EDTA. EDTA disodium-0.01 g, d. water-10 ml. Dissolve ingredients and adjust the pH to 13 for 0.1% EDTA. EDTA disodium-0.1 g, d. water-10 ml. Dissolve ingredients and adjust the pH to 13 for 1% EDTA. EDTA disodium-1 g, d. water-10 ml. Dissolve ingredients and adjust the pH to 10 for 10% EDTA.

14) SDS solution-SDS-0.25 g, phosphate buffer-5 ml. Dissolve SDS in phosphate buffer (pH 1-13) for 5% SDS solution.

Vita

Jiemin “Dove” Cormier was born in June 1987, in Wuhan, China. She earned her Bachelor of Science degrees in Biotechnology and Chemistry in 2009 from Central China Normal University, Wuhan, China. She then started working her Master of Science degree in Food Science in 2010 from Department of Food Science, Louisiana State University, Baton Rouge, Louisiana and transferred to a PhD program in the same department from fall of 2012. Currently, she is a candidate for the degree of Doctor of Philosophy in food science in the College of Agriculture. She will receive her doctoral degree in fall 2013.

During her graduate career, she has been actively involved in multiple research projects and an active member in the International Association for Food Protection. She has given two poster presentations and one technical presentation at the International Association for Food Protection Annual Meetings, and one technical presentation at the Trans-Atlantic Fisheries Technology Conference. She was nominated for the International Association for Food Protection Developing Scientist Award in 2013.