DISSERTATION

RESIN-BASED METHOD FOR CONCENTRATION OF ENTERIC VIRUSES AND F-RNA COLIPHAGES FROM WATER SAMPLES

Submitted by

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ABSTRACT

RESIN-BASED METHOD FOR CONCENTRATION OF ENTERIC VIRUSES AND F-RNA COLIPHAGES FROM WATER SAMPLES

Fecal contamination of source and recreational waters represents a public health concern due to potential content of human pathogens, and the variety of sources from which an individual may be exposed to such contamination. Enteric viruses such as noroviruses, rotaviruses, adenoviruses and hepatitis viruses are dispersed by fecal contamination and are a major cause of waterborne diseases in the US and worldwide. Given the variety of viral enteric pathogens and their particular growth requirements, their detection is technically difficult and time consuming. An alternative to determine the risk of enteric virus contamination in water is to detect viral indicators of fecal contamination. F-RNA coliphages are recognized as enteric virus surrogates, fecal indicators useful for source tracking. Enteric viruses and F-RNA coliphages are often present at low concentrations in contaminated waters; therefore rapid, sensitive and cost effective viral concentration methods applicable to different environmental water samples are needed for an accurate assessment of water microbiological safety.

Here, a resin-based virus concentration method was developed and tested. The method is based on adsorption of the viruses to an anion exchange resin dispersed in the water sample, followed by direct isolation of nucleic acids from the resin to provide a small volume final sample. In order to test the method with a wide variety of viral structures and characteristics, three enteric viruses (hepatitis A virus, adenovirus and

rotavirus) and four F-RNA coliphages were used. Additionally, tap water and a variety of environmental samples were tested. After virus concentration, detection was performed through real time RT-PCR, a sensitive molecular technique widely use for detection of these viruses.

In tap water containing 10^5 pfu/ml of F-RNA coliphages, the anion exchange resin adsorbed over 96% of the coliphage present, allowing for detection of between 10^0 to 10^{-1} pfu/ml of F-RNA coliphages in 50 ml samples. Similarly, experiments with large volumes of tap water showed that the resin-based method was capable of detection limits as low as 10 $TCID_{50}$ of enteric viruses in tap water. Finally, the evaluation of the method with different samples of environmental water showed that the resin was useful for concentration of F-RNA coliphages in most of the samples, despite the presence of PCR inhibitors in the water. Limitations of the method included incomplete recovery of nucleic acids from the resin and concentration of PCR inhibitors from the samples. Given the simplicity of the method and the promising results obtained in this work, studies focused in increasing the yield of nucleic acids and decreasing the concentration of environmental inhibitors in the concentrated sample is warranted.

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Chapter 1

Literature Review Part I: Enteric Viruses

1.1 Foodborne viruses and illnesses

Enteric viruses include a wide variety of viruses that infect the cells lining the intestinal tract of humans (Bosch, 1998; Koopmans and Duizer, 2004; Schultz et al., 2011). They are dispersed by shedding in very high numbers into the stool or through emesis (vomiting), and are transmitted to humans through contaminated food and water, or through person-to-person contamination (Richards, 2001). Enteric viruses are highly infective, requiring the fewest number to cause infection of all foodborne microorganisms (Reynolds et al., 2008) with infectious doses ranging from 1 to 100 viral particles (Appleton, 2000; Bresee et al., 2002; Ikner et al., 2011; Koopmans and Duizer, 2004). Viruses are very small microorganisms, from 15 to 300 nm. Enteric viruses cause a wide range of diseases, from mild gastroenteritis to life threatening conditions such as hepatitis and meningitis (Koopmans and Duizer, 2004).

In the US, the noroviruses account for 58% of domestically acquired foodborne illnesses that are caused by a known agent. In addition, the norovirus contribute to 26% (14,663) of the domestically acquired foodborne illnesses resulting in hospitalization and to 11% (149) of the acquired foodborne illnesses resulting in death (Scallan et al., 2011). The number of enteric virus illnesses in the United States can only be estimated because most of the illnesses are mild, go unreported, and routine testing of patients for specific virus infections is not performed (Richards, 2001).

The most important enteric viruses and their associative illness include norovirus (diarrhea), rotavirus (diarrhea), hepatitis A virus (hepatitis, liver damage), and adenovirus (diarrhea, respiratory disease, hearth disease, eye infections). Other viruses known to cause waterborne and foodborne illness include enterovirus, astrovirus, poliovirus, hepatitis E virus, reovirus, coronavirus, echovirus, paraechovirus, sapovirus and coxsackievirus (Bosch et al., 2008; Reynolds et al., 2008). Noroviruses (NoV) and hepatitis A virus (HAV) are by far the most common cause of illness by this mode of transmission. Still, some large foodborne outbreaks have occurred with group B and C rotaviruses, and waterborne outbreaks have occurred with hepatitis E virus (Koopmans and Duizer, 2004).

1.1.1 Norovirus

Taxonomically, the NoVs are a member of the family *Caliciviridae,* a group of non-enveloped icosahedral single-stranded RNA virus. There are five recognized norovirus genogroups, of which GI, GII, and GIV are known to affect humans. Within these genogroups, more than 25 different genotypes have been identified (Karst, 2010). Noroviruses have been reported in developing and industrialized countries of the five continents (Patel et al., 2008). NoV outbreaks frequently occur in semi-closed communities such as cruise ships, nursing homes, schools, hospitals, military settings, and disaster relief situations. Norovirus infection presents acute-onset vomiting, watery non-bloody diarrhea with abdominal cramps, and nausea. Recovery is usually complete and there is no evidence of any serious long-term sequelae. Throughout the

world, these viruses cause at least 95% of nonbacterial gastroenteritis outbreaks, and 50% of all gastroenteritis outbreaks (Karst, 2010).

1.1.2 Rotavirus

Rotavirus (RV) is the most important causal agent of infantile diarrhea; it has been estimated that more than 600,000 rotavirus related infant deaths occur worldwide each year (Parashar et al., 2006). In the United States, relatively few childhood deaths are attributed to rotavirus (approximately 20–60 deaths per year among children younger than 5 years of age), nonetheless, rotavirus causes an estimated 2.7 – 3.9 million illnesses worldwide and 49,000 – 50,000 in the US (CDC, 2008; Richards, 2001). This virus belongs to the family *Reoviridae*, genus *Rotavirus*, and it has been detected in drinking water and linked to rotavirus outbreaks (Gratacap-Cavallier et al., 2000). The viral nucleocapsid is composed of three concentric shells that enclose 11 segments of double-stranded RNA. The outermost layer contains two structural viral proteins (VP) that define the serotype of the virus and are considered critical to vaccine development (King, 2012). Severe watery diarrhea, vomiting, fever, and abdominal pain characterize rotavirus illness, also referred to as acute infantile diarrhea. In babies and young children, it can lead to severe dehydration, particularly in regions of the world where rehydration therapy is not available (CDC, 2010b; Richards, 2001).

1.1.3 Hepatitis

Hepatitis refers to a group of viral infections that affect the liver. The most common types are Hepatitis A, Hepatitis B, and Hepatitis C. Two other types (hepatitis D and E) are unusual in the US. Hepatitis B, C and D are transmitted by blood, percutaneous and mucosal contact, while Hepatitis A (HAV) and Hepatitis E (HEV) viruses are foodborne pathogens, transmitted by the oral-fecal route or by ingestion of contaminated food and water (CDC, 2010c). Worldwide, clean drinking water is an inverse predictor of HAV infection rates (Jacobsen and Koopman, 2005). HAV is often associated with raw or lightly cooked shellfish, or uncooked HAV contaminated foods (Costafreda et al., 2006). For example, the largest foodborne outbreak of hepatitis A was linked to consumption of contaminated clams in Shanghai, China, where nearly 300,000 people became sick during a 2-month period (Xu et al., 1992). In the US, hepatitis A virus causes an estimated 83,000 illnesses per year, is often self-limiting and spontaneous recovery is common. Symptoms include those typical for other acute viral diseases (fatigue,nausea, vomiting, fever, anorexia) in addition to abdominal pain, dark urine, clay-colored bowel movements and jaundice (CDC, 2010c). Hepatitis A virus (HAV) is the only species of the genus *Hepatovirus,* family *Picornaviridae* (King, 2012).

1.1.4 Adenovirus

Human adenoviruses (HAdV) belong to the Family *Adenoviridae*, genus *Mastadenovirus* and comprise 54 serotypes classified in seven species. The two members of the species *Human adenovirus* F or enteric adenoviruses (HAdV-40 and HAdV-41) are present in high amounts in feces of young children with acute

gastroenteritis, and are second only to rotaviruses as a major cause of infantile viral diarrhea (King, 2012). The illness is usually mild and self-limiting but can be persistent and severe in the immunosuppressed, causing specific adenovirus colitis in HIV infected patients. Although epidemiologic characteristics of the adenoviruses vary by type, all are transmitted by direct contact, fecal-oral transmission, and occasionally waterborne transmission (Jiang, 2006). Some types of HAdV can establish persistent asymptomatic infections in tonsils, adenoids, and intestines of infected hosts, and shedding can occur for months or years (CDC, 2010a; Clark and MacKendrick, 2004). As adenoviruses are very persistent in water, they are conservative indicator for human viral fecal contamination (Jiang, 2006; Mena and Gerba, 2009).

1.2 Routes of food contamination

There are two main points at which food may become contaminated with enteric viruses including at the site of production (pre-harvest contamination) or during post-harvest processes as picking, washing, packing, transportation, cooking and serving.

Pre-harvest enteric virus contamination occurs thorough contact of contaminated irrigation or run off water with fruits and vegetables during production and with contaminated water in growing areas for shellfish production. Pre-harvest contamination of fruits and vegetables occur if fecal contamination is present in the field as consequence of irrigation with reclaimed wastewater or crop fertilization with sewage sludge (Richards, 2001). Several outbreaks of hepatitis A and viral gastroenteritis

linked to salad items and soft fruits, are believed to have been caused by produce contaminated at their source (Koopmans and Duizer, 2004).

Sewage and discharge of human wastes from boats and land runoff into watersheds are important sources of contamination for shellfish, and these events have led to foodborne illnesses (Richards, 2001). For example, a large, multistate outbreak of illness associated with oysters was traced back to a sick oyster harvester who had vomited and disposed of the waste overboard (Koopmans and Duizer, 2004). The bivalve mollusks feed by filtering large volumes of water, in this way they can concentrate up to 100-fold pathogens that occur within the surrounding water (Appleton, 2000).

Post-harvest contamination of food may occur by contact with (human) feces, fecally soiled materials (including hands) or fecally contaminated water; by contact with vomit or water contaminated with vomit, or by contact with aerosols generated by infected people and/or environments in which infected people were present (Koopmans and Duizer, 2004). In addition, enteric virus outbreaks have been associated with contamination from the hands of food handlers, recontamination after cooking or processing, and inadequate sanitation (Richards, 2001). Cold items, such as sandwiches and salads that require much handling during preparation, are frequently implicated (Appleton, 2000). Cross-contamination of processed products by uncooked food has also been reported, such as the contamination of salad by raw seafood. Celery contaminated with non-potable water was responsible for an outbreak of Norovirus gastroenteritis, causing morbidity in over 1400 people. Ice may become

contaminated during or after production and can lead to food or waterborne illness (Richards, 2001).

1.3 Food processing interventions

Most foodborne and waterborne viruses are more resistant to heat, disinfection and pH changes than most bacteria, and survive extremely well in the environment (Bosch, 1998). Thorough cooking is one of the best and most practical methods to totally inactivate enteric viruses; however, high protein and fat content tends to enhance thermal stability of enteric viruses. Both the noroviruses and hepatitis A virus retain infectivity after heating to 60°C for 30 min (Appleton, 2000). HAV is not inactivated in pasteurization processes of 70ºC for 2 minutes or 71.7ºC 15 seconds (Koopmans and Duizer, 2004). Studies performed by adding poliovirus to oysters followed by stewing, frying, baking, and steaming showed that although different cooking methods gave different results, 7% to 13% of the inoculated virus remained active (Richards, 2001).

Irradiation is another process used in the food industry for reducing pathogen levels, and it is effective in reducing enteric viruses in foods. Nevertheless, gamma irradiation doses needed to inactivate viruses are higher than doses needed to inactivate pathogenic bacteria. Rotavirus and poliovirus demonstrated similar rates of inactivation from UV; requiring three to four times the dose of UV necessary for *Escherichia coli* inactivation (Richards, 2001).

Viruses that infect via the gastrointestinal tract are acid stable. Both the noroviruses and hepatitis A virus retain infectivity after exposure to high acidity levels.

Norovirus resists acidification to pH 2.7 for 3 hrs, while HAV resists 5 hrs in pH 1 (Richards, 2001). Both viruses survive food processing and preservation conditions designed to produce the low pH that inhibits bacterial and fungal spoilage organisms (e.g. pickling in vinegar and fermentation processes). Outbreaks of viral gastroenteritis have been associated with cockles pickled in brine and vinegar (Appleton, 2000).

Most enteric viruses remain infectious after refrigeration and freezing. Frozen foods that have not received further cooking have been implicated in a number of incidents of both viral gastroenteritis and hepatitis A (Appleton, 2000), as is demonstrated by the recent multistate outbreak of hepatitis A virus infection linked to pomegranate seeds from Turkey (CDC, 2013).

1.4 Prevention

Given the high amount of viral shedding by infected people, the environmental resistance of the enteric viruses, the low doses needed for infection and the lack of efficient viral detection methods in food, the best way to avoid foodborne viral illnesses is through prevention.

Clean water would be the most important tool for preventing pre-harvest contamination of crops and shellfish. Important risk factors are sewage pollution of water sources and sewage sludge applied to agricultural land, not only because possible contamination of produce produced there, but because viruses from sewage deposited on land enter ground waters, leading to contamination of water sources (Appleton, 2000). To prevent post-harvest contamination all steps of washing and

preparation should be done with water of drinking quality, and personnel handling and preparing foods should follow strict hygiene practices (Appleton, 2000; Koopmans and Duizer, 2004; Richards, 2001).

Chapter 2

Literature Review Part II: F-RNA Coliphages and Source Tracking

2.1 Indicators of fecal contamination and source tracking

The microbiological safety of drinking water constitutes one of the most important tools to prevent morbidity and mortality due to diarrheal diseases all around the world. The potential of contaminated water to cause illness to great numbers of people is well documented in countries at all levels of economic development (WHO-OECD, 2003). The microorganisms that can be found in contaminated water include hundreds of enteric bacterial, viral and parasitic pathogens (Figueras and Borrego, 2010), whose diverse nature requires a wide variety of microbiological approaches for their detection. Analysis for individual pathogen presence in water is costly, difficult, not effective and consequently not in the best interest of public health. By the end of the 19th century, Koch postulated that the presence of heterotrophic bacteria, quantified by colony count, was a measure of pollution. Once the importance of fecal contamination transmission of pathogens was established, and owing to the fact that the majority of pathogens are fecally derived, public health official decided that monitoring for the presence of indicator microorganisms would be the best way to detect fecal contamination (Leclerc et al., 2000; WHO-OECD, 2003), Thus, the concept of fecal indicator microorganisms was proposed.

The notion of examining microbial indicators of fecal pollution continued to be developed. *Escherichia coli* became the primary indicator of fecal pollution and soon

other Gram-negative, lactose-fermenting bacteria were isolated from stools and water. *E. coli* and its relatives, the total coliforms, became the most widely used indicator of fecal contamination, being rapid, inexpensive, and easy to detect. By 1970, it was clear that many other bacterial species that meet the coliform definition are not related to fecal contamination, and they are also able to multiply in the aquatic environment, thus reducing their value as an indicator of fecal contamination (WHO-OECD, 2003). Additionally, more and more information has accumulated about how the ecology, prevalence and resistance to stress of the coliform bacterial group differ from many of the pathogens they intend to represent, limiting even more the utility of coliforms as indicator microorganisms (Leclerc et al., 2000; Scott et al., 2002). Other bacteria such as *Enterococcus* spp. and *Clostridium perfringes* have proven to have certain value as indicators of fecal contamination, but similar shortcoming as the ones indicated by coliforms (regrowth in the environment and different prevalence and tolerance to the environment from other pathogens) have restricted their use (Scott et al., 2002).

Fecal coliforms and enterococci do not provide information about the source of fecal pollution, however, identifying dominant sources of fecal pollution is critical for accurate assessment of public health risks and implementation of proper interventions and best management practices (Wong et al., 2012). In order to determine the origin of fecal contamination that influences a water body, microbial source tracking (MST) techniques, including chemical and microbiological (both genotypic and phenotypic methods), have been developed (Noble et al., 2003; Scott et al., 2002). The rationale behind the source tracking approach is to identify groups of chemical compounds or microorganisms strongly related to specific animal or human hosts, which can be

subsequently used to identify the host or environment from which they were derived (Scott et al., 2002). Chemical compounds such as fecal sterols and caffeine have been proposed as chemical indicators of human or animal fecal contamination. Microbial methods include direct monitoring for human pathogens including enteric viruses and parasites, fecal coliform/fecal streptococcus ratio, presence of *Bifidobacterium* spp, *Rhodococcus coprophilus* and certain phenotypes of *Bacteroides* species. Phenotypic methods such as antibiotic resistance analysis, carbon utilization profile and immunological methods have been use to differentiate groups of bacteria from the same species that are associated with specific hosts. In the same way, genotypic (subtyping) methods including pulse field gel electrophoresis (PFGE), ribotyping, length heterogeneity PCR, terminal-length restriction fragment polymorphism and repetitive PCR have been used to differentiate between closely related bacteria but associated to different hosts or environments (Schaper et al., 2002a; Scott et al., 2002; Simpson et al., 2002; Wilkes et al., 2013).

2.1.1 Viral indicators of fecal contamination

Waterborne outbreaks related to potable water that met current standards for coliforms and disinfectant residual demonstrated that it is unsafe to rely on bacteriological standards to assess the virological quality of any kind of water. For example, during an investigation of an outbreak of infectious hepatitis; HAV, rotaviruses and enteroviruses were detected in water samples that showed adequate levels of chlorine and were free of indicator bacteria (Bosch, 1998; Sobsey et al., 1990). This

kind of evidence and increasing awareness of the shortcomings of bacteria indicators triggered the search for fecal indicators with similar resistance and behavior as enteric viruses.

Currently, both pathogenic and non-pathogenic viruses are used as fecal indicators and for source tracking. Due to their host specificity and prevalence, pathogenic viruses such as adenovirus, poliovirus, echovirus, coxsackievirus and bovine and porcine enteroviruses have been used as tracers of human or animal fecal contamination (Jiang, 2006; Noble et al., 2003; Wong et al., 2012). On the other hand, non-pathogenic prokaryotic viruses as bacteriophages (phages) are attractive alternative indicators for fecal pollution. Due to the fact that phages share structural features with enteric viruses, they may reflect the behavior and resistance to treatment process of viruses much closer than bacterial indicators. Additionally, phages are detectable by simple and inexpensive techniques that yield results in a relatively short period of time and do not constitute a health risk to laboratory workers (Jofre et al., 2011).

2.1.1.1 Phages infecting *Bacteroides*

Bacteroides is a genus of obligated anaerobic Gram-negative bacteria that constitute a substantial portion of the gastrointestinal flora of the mammals. Bacteriophages infecting strains of *B. fragilis, B. thetaiotaomicron, B. ruminicola*, and *B. ovatus* have been detected in feces and wastewater. Evidence suggests that these

tailed bacteriophages belong to the *Siphoviridae* family, possess a genome of double-stranded DNA and infect the host through the cell wall.

Most *Bacteroides* phages have a narrow host range, and strains of *Bacteroides* spp. differ in the numbers of phages that they recover from sewage and in their ability to detect bacteriophages in the fecal material of different animal species, including humans. This characteristic has been used to discern the fecal source that contaminates a given sample (Puig et al., 1999). Thus, strain RYC2056 and VPI3625 of *B. fragilis* detect phages both in human and nonhuman fecal wastes whereas *B. fragilis* HSP40, *B. thetaiotaomicron* GA17 and *B. fragilis* GB124 detect phages mostly in human fecal wastes. Bacteriophages infecting *B. fragilis* have been reported to have similar resistance to heat, UV light and chemical disinfectants as enteric viruses and other bacteriophages, higher resistance than bacterial indicators of fecal contamination. Probably the most important shortcomings of these bacteriophages as routine viral fecal indicators are the very low concentration in polluted environments and the very special growth requirements of their bacterial hosts (Puig et al., 1999; Scott et al., 2002).

2.1.1.2 F-RNA coliphages

The family *Leviviridae* consists of a group small (25 to 28 nm) phages. They have single stranded positive sense RNA genomes that encode for only four proteins, icosahedral capsid, and share structural characteristics with pathogenic human enteric viruses including caliciviruses, hepatitis A and E viruses, enteroviruses and astroviruses (Jofre et al., 2011). This family of phages infect *E. coli* and other close related bacteria

that produce an F pilus, structure that serves as the site of attachment for the virus and is expressed only at temperatures above 30C (Grabow, 2001). Due to this characteristic, these phages are called F-RNA coliphages (Scott et al., 2002). The F-RNA coliphages do not proliferate in environments other than the gastrointestinal tracts of warm-blooded animals, are shed exclusively in feces, share structural features with enteric viruses and display similar chemical/environmental sensitivities (Grabow, 2001; Havelaar et al., 1993). These factors make F-RNA coliphages exceptional indicators of fecal contamination as well as enteric virus model organisms (Charles et al., 2009; Ikner et al., 2011; Jofre et al., 2011; Lukasik et al., 2000; Zerda et al., 1985). For example, F-RNA coliphages are useful indicators of norovirus and other enteric viruses in molluscan shellfish and fresh water (Doré et al., 2000; Havelaar et al., 1993). The F-RNA coliphage MS2 was used as a surrogate for human enteric viruses in studies of wastewater disinfection and viral persistence in surface water and ground water (Bae and Schwab, 2008; Tree et al., 2005) and F-RNA coliphages have been used as indices and indicators of viral contamination on animal carcasses (Flannery et al., 2009; Jones and Johns, 2012). The United States Environmental Protection Agency recognizes F-RNA coliphages as one of the three groups of microorganisms useful as fecal indicators (EPA, 2000).

Based on their serological and genetic properties, F-RNA coliphages are classified into four genogroups within two genera: genogroups I and II are comprised within the genus *Levivirus*, and genogroups III and IV within the genus *Allolevivirus* (Leclerc et al., 2000). The presence or absence of particular F-RNA coliphage genogroups can be used to track the origin of fecal pollution. F-RNA coliphage

genogroups II and III are primarily associated with human sources of fecal contamination, while genogroups I and IV are predominantly associated with animal feces (Schaper et al., 2002b). However, some reports indicate that F-RNA coliphage/host associations are not absolute. Genogroups II and III were found in poultry, cattle, swine and dog feces, and genogroups I and IV have been detected in human waste water (Jofre et al., 2011; Noble et al., 2003; Scott et al., 2002). Despite specificity issues, it has been demonstrated that using statistical tools (principle coordinate analysis) applied to the information of genotype presence and genotype clusters of F-RNA coliphages (Lee et al., 2011; Lee et al., 2009), it was possible to differentiate the fecal origins from human and animals (Wong et al., 2012).

Chapter 3

Literature Review Part III: Virus Concentration

3.1 The challenge

The lack of standard and reliable methods to concentrate, detect and isolate low concentrations of viruses from large volumes of water is one of the major problems facing environmental health officials concerning water quality control (Hill Jr et al., 1971). Effective and sensitive methods of detection are needed to assess the viral quality of the water for monitoring purposes, risk assessment studies and design of better sampling and intervention strategies. Viral pathogens and viral indicators are present in the water environment at low concentrations. For example human adenovirus, reported to have higher density than other enteric viruses in wastewater associated samples, has been found in river water at concentrations as low as 0.9 genome copies $\log_{10}$ /L (Wong et al., 2012); therefore, viral concentration procedures are needed to facilitate detection of the target, even when sensitive modern molecular techniques are used. Water matrixes are highly variable in different zones, seasons and environments, a fact that introduces important technical challenges for development of concentration and detection methodologies.

Despite repeated efforts and decades of research, there is no ideal method that gives consistently high recoveries and adequate sensitivities, applicable to a broad range of viruses and water matrixes (Ikner and Gerba, 2012; Lambertini et al., 2008; Victoria et al., 2009). Ideally, any viral concentration method must: (i) be technically

easy and not time consuming;(ii) have a high virus recovery rate;(iii) be useful for a large range of viruses;(iv) provide a small volume of concentrate free of inhibitors for downstream detection techniques;(v) be cost-effective;(vi) be capable of processing large volumes of water;(vii) and be repeatable and reproducible (Wyn-Jones and Sellwood, 2001).

3.2 Virus concentration methods

Several methods have been developed for virus concentration, using diverse strategies that explore different structure characteristics of the virions.

3.2.1 Concentration based on colloidal nature

Because of their colloidal nature and interaction with the dispersion medium, viruses can be concentrated by flocculation, when a change in salt concentration, pH or addition of charged polymers to the sample is used to promote viral aggregation and consequent precipitation (Safferman et al., 1988). Once precipitated, the virus is recovered by centrifugation and resuspended in a small volume of diluent. This method shows relatively low recovery efficiency and is not suitable for large volume samples. Additionally, the method is limited by reagent added to promote flocculation that produce occasional toxicity for cell culture or molecular detection inhibition (Grabow, 2001; Wyn-Jones and Sellwood, 2001).

3.2.2 Concentration based on particle size

Other virus concentration methods such as ultracentrifugation, ultrafiltration and hydro-extraction, take advantage of the viral particle size to concentrate viruses by size exclusion or entrapment. Ultracentrifugation is used to pellet viruses from the sample, followed by suspension of the pellet in a small volume of diluent, allowing for highly efficient virus concentration. Due to the small size of viral particles (20 to 300nm), centrifugation forces higher than 100,000 ×g are required for successful recovery of the targets, limiting the application of this concentration method to small volumes of water and costly equipment (Ikner and Gerba, 2012). Ultrafiltration is used to retain virus-size particles in small pore size membranes, usually excluding particles from 5 to 100 kiloDaltons. Larger virus and particle cannot pass through the membrane and are retained on the filter and or in a small volume of the feeding solution. Recovery of the virus from the membrane is generally accomplished by elution or back flushing of the filter using a small volume of diluent. This concentration method is costly and not amenable for field applications (Ikner and Gerba, 2012). In hydro-extraction, a dialysis membrane is used to retain the virus while water is extracted from the sample by passive diffusion. In this method, as in ultracentrifugation and ultrafiltration, concentration of other particles, frequently inhibitors for the downstream detection method, is the major concern (Wong et al., 2012).

3.2.3 Concentration based on surface electrostatic charge

Due to their unique surface properties, virus adsorb to a variety of materials (Hill Jr et al., 1971), primarily by electrostatic and hydrophobic interactions (Gerba, 1984). This phenomenon has enabled the development of a variety of virus concentration methods based on the use of different materials for virus adsorption and subsequent elution. These materials include ion exchange resins (Muller, 1950), gauze pads (Kelly, 1953), polyelectrolites (Wallis et al., 1971), glass powder (Schwartzbrod and Lucena-Gutierrez, 1978), fiberglass (Joret et al., 1980), glass wool (Lambertini et al., 2008) and charged filters (Goyal et al., 1980; Logan et al., 1980; Lukasik et al., 2000; Sobsey et al., 1990; Victoria et al., 2009) among others. Currently, electronegative and electropositive filters (in membrane or cartridge formats) are the most common systems used for viral concentration purposes (Ikner and Gerba, 2012). As viruses exhibit amphoteric properties, their net surface electric charge changes depending on their isoelectric point (pI) and environment pH, as their interaction with solid surfaces changes as well (Zerda et al., 1985). When the pH is above the isoelectric point of the capsid, the virus exhibits a net negative charge and adsorbs to electropositive filters. On the contrary, when the pH is below the viral capsid isoelectric point, it is adsorbed to electronegative filters because of its positive charge. As most enteric viruses are negatively charged at neutral pH (Michen and Graule, 2010), electropositive filters (zeta-plus filter, virosorb 1-MDS cartridges, nylon membranes and cartridges, triple-layered PVDF filters and argonide nanoceram cartridge and disk filters) are used for virus adsorption. However, electronegative filters (millipore cellulose nitrate membrane filters and fiberglass-epoxy filters and cartridges) have been widely used through

sample conditioning, where the pH of the sample is brought to low levels (3.5) to promote positive charges on the virus surface and maximize virus interaction with the filter (Ikner and Gerba, 2012; Wyn-Jones and Sellwood, 2001). Once the virus is captured on the filter surface, concentration is accomplished by eluting adsorbed virions in a small volume of eluent. The elution process, besides being frequently inefficient, utilizes extreme pH or high protein content solutions that have been reported to inactivate the eluted viruses or inhibit downstream molecular detection methods (Ikner and Gerba, 2012). Additionally, the large volume of eluent (depending on the size of the filter, from 10 to 300 ml, or in some methods up to 1,2 L) is not practical for molecular detection techniques as PCR, because only a small fraction of the concentrated sample can be analyzed in a conventional reaction (Ikner and Gerba, 2012; Wu et al., 2011). To overcome this shortcoming, these adsorption methods frequently require a secondary concentration step (ultracentrifugation, ultrafiltration, flocculation, evaporation), lowering the overall recovery efficiency of the method and increasing its complexity, time and cost (Ikner and Gerba, 2012; Wu et al., 2011; Wyn-Jones and Sellwood, 2001).

3.3 Research summary

In this project, we propose a novel method to concentrate viruses from water, based on the use of an anion exchange resin (IRA-900) as an alternative to electropositive filters. Anionic exchange resins are insoluble organic polymers containing cation groups covalently linked. These positively charged groups attract and

hold anions present in a surrounding solution in exchange for anions previously held. Each resin bead is a 0.5 to 1.0 mm sphere with a porous surface; characteristic that increases enormously the exchange surface. In the proposed method, this resin is dispersed in the water sample to facilitate the adsorption of negatively charged viruses, providing an alternative concentration format that overcome clogging problems, a frequent issue in filter based systems. Additionally, as an option to cumbersome and inefficient elution steps, direct isolation of viral nucleic acid from the viruses adsorbed on the resin is proposed, an alternative that can be performed using small volume of reagents resulting in final small volume samples. The detection system used in this work is real time PCR, a sensitive and quantitative technique widely used in virology, but susceptible to environmental inhibitors. In this way, the proposed concentration system is challenged to produce nucleic acid samples compatible with a molecular detection technique of general use and in some cases commercially available. To test this methodology, four different F-RNA coliphages and three enteric viruses were used, providing examples of viruses with different structure, size, nucleic acid (RNA and DNA) and isoelectric points, including viral indicators and viral pathogens of public health importance. Different volumes and origin of samples were also used, in order to test the applicability of the method to different field applications.

3.4 The results

The results obtained testing this methodology were organized in three different chapters, each one prepared as a journal manuscript. In chapter 4 results of small

volume (50 ml) laboratory experiments concentrating F-RNA coliphages spiked in tap water are presented. In chapter 5 the methodology is tested using a diverse panel of 1 L water environmental samples, measuring the performance of the method to concentrate spiked and naturally present F-RNA coliphages. In chapter 6 results of experiments with large volumes of tap water (10 L) spiked with human adenovirus, rotavirus and hepatitis A virus are presented, and the compatibility of the concentration method with commercially available kits for virus detection was evaluated. Finally, in chapter 7 results of experiments on stability of F-RNA coliphages in filter paper are presented. Although not related to the virus concentration methodology, these experiments provided valuable information for the handling and shipment of F-RNA coliphages.

Chapter 4

Evaluation of an Anion Exchange Resin-based Method for Concentration of F-RNA coliphages (Enteric Virus Indicators) from Water Samples

4.1 Introduction

There are more than 140 enteric viruses excreted in human and animal wastes (Fong and Lipp, 2005; Leclerc et al., 2000), and water is recognized as one of the most important vehicles for their transmission (Kelly, 1953). Individuals may be exposed to such contamination through drinking water, recreational water, irrigation and food processing water, or seafood produced in impacted environments (Bosch et al., 2008). Thus, efficacious strategies for enteric virus detection in water are needed to assess potential health hazards, assist epidemiological investigations, understand the ecology and transmission of different viruses and to measure the effectiveness of water treatments (Bosch et al., 2008; Wyn-Jones and Sellwood, 2001). Ideally, such a method would be sensitive, rapid, reliable, inexpensive, compatible with different water types and technically feasible for a large number of samples.

Given the variety of viral enteric pathogens and their particular growth requirements, detection of all possible enteric viruses in a water sample is technically challenging, time consuming and expensive (Leclerc et al., 2000). An alternative to determine the risk of enteric virus contamination of water is to detect viral indicators of fecal contamination, such as F-RNA coliphages. These coliphages are members of the family *Leviviridae*, have single stranded positive sense RNA genomes and are non-

pathogenic for humans (Jofre et al., 2011). In nature, F-RNA coliphages are shed exclusively in feces (Grabow, 2001; Havelaar et al., 1993), share structural features with enteric viruses and display similar chemical/environmental sensitivities. These factors make F-RNA coliphages exceptional indicators of fecal contamination as well as enteric virus model organisms (Charles et al., 2009; Ikner et al., 2011; Jofre et al., 2011; Lukasik et al., 2000; Zerda et al., 1985). For example, F-RNA coliphages are useful indicators of norovirus and other enteric viruses in molluscan shellfish and fresh water (Doré et al., 2000; Havelaar et al., 1993). The F-RNA coliphage MS2 was used as a surrogate for human enteric viruses in studies of wastewater disinfection and viral persistence in surface water and ground water (Bae and Schwab, 2008; Tree et al., 2005).

Enteric viruses and their viral indicators are often present in naturally contaminated water at such low concentrations that they are undetectable without upfront concentration, even when modern molecular methods such as quantitative real time PCR are utilized. Several methods exist to concentrate viruses from water, which exploit morphological features of the virion(s). These features include viral colloidal nature, particle sizes and the chemical and physical properties of the virion surface, most notably electrostatic charge (Ikner and Gerba, 2012; Wyn-Jones and Sellwood, 2001). Electronegative and electropositive filters (in membrane or cartridge formats) are currently the most commonly used viral concentrators for water analysis (Ikner and Gerba, 2012). Once viruses are adsorbed on the filter surface, concentration is accomplished by eluting adsorbed viruses. The elution process, which is often inefficient, utilizes extreme pH or high protein content solutions, which have been

reported to inactivate the target virus and/or inhibit downstream detection (Grabow, 2001; Ikner and Gerba, 2012). Additionally, 10 to 300 ml of buffer are required for elution of the filters, and as molecular detection techniques are intended to analyze very small volumes of sample (5 to 10 µl per reaction), this process reduces the sensitivity of downstream molecular detection and necessitates a secondary concentration step, increasing processing time and cost (Ikner and Gerba, 2012; Wu et al., 2011; Wyn-Jones and Sellwood, 2001).

Alternative materials have been used for virus adsorption including ion exchange resins (Muller, 1950), gauze pads (Kelly, 1953), polyelectrolites (Wallis et al., 1971), glass powder (Schwartzbrod and Lucena-Gutierrez, 1978) and fiberglass (Joret et al., 1980). Nonetheless, when these materials were tested, molecular techniques were not developed, and their applicability was limited to culture-based techniques for virus detection.

Virus concentration methods should be applicable to a large range of viruses, different water matrices, have a high recovery rate, be easy to perform, cost effective and rapid. Additionally, such methods should provide a small volume of concentrate free of inhibitors that can complicate downstream detection (Bosch et al., 2008; Grabow, 2001; Wyn-Jones and Sellwood, 2001). In this study, we propose a method to concentrate viruses from water using an anion exchange resin (IRA-900) dispersed in the sample to facilitate the adsorption of negatively charged viruses. Isolation of viral nucleic acids can be performed directly from the resin, without the need for large-volume elutions, with the resulting sample compatible with real time RT-PCR detection. To test this methodology, four different F-RNA coliphages (each belonging to one of the

four F-RNA genogroups) were used, providing examples of viruses with similar structures to enteric viruses and having diverse isoelectric points (pI) ranging from 2.1 to 5.3 (Michen and Graule, 2010). This system proved to be effective for F-RNA coliphage adsorption and detection, providing an alternative to filter-based concentration methods.

4.2 Materials and methods

4.2.1 Coliphages and bacterial strains

F-RNA coliphages from genogroup I (MS2 ATCC 15597-B1), genogroup III (Qβ ATCC 23631-B1) and the bacterial host E. coli HS (pFamp)R (ATCC 700891) were obtained from the American Type Culture Collection (Manassas, VA). F-RNA coliphage GA (genogroup II) and HB-P22 (genogroup IV) were kindly provided by Stephanie Friedman (US EPA, Gulf Breeze, FL, USA). Coliphages were propagated by infection of a logarithmic phase *E. coli* culture in tryptic soy broth supplemented with 5 mM magnesium chloride and 50 µg/ml of ampicillin and streptomycin (Sigma-Aldrich, Saint Louis, MO), at a multiplicity of infection of 5-10. After 18 to 24 hr of incubation with shaking at 37°C, coliphage stocks were prepared by adding 10% v/v chloroform to the infected bacterial culture, followed by centrifugation at 6,000 × g for 25 min at 4°C to remove cellular debris. The supernatants were filtered through a 0.22 µm low protein-binding filter (PALL Life Sciences, Ann Arbor, MI) and stored at 4°C. Coliphage stocks were regularly enumerated using the double agar overlay plaque assay (Hershey et al., 1943) as modified by Kropinski et al. (2008).

4.2.2 Adsorption of F-RNA coliphages to anion exchange resin

Tap water from the Fort Collins, CO municipal water supply was dechlorinated using 0.05 g/L of sodium thiosulphate pentahydrate (Mallinckrodt Baker Inc. Phillipsburg, NJ). Serial ten-fold dilutions of coliphage stocks were prepared in lambda buffer [0.58% NaCl, 0.2% MgSO4 heptahydrate, 0.01% gelatin, and 0.05 M Tris-HCl, pH 7.5 (Sigma-Aldrich)] and 1 ml of the appropriate dilution was used to inoculate 50 ml water samples (final coliphage concentrations ranging from 10^{-1} to 10^{5} pfu/ml).

To determine the adsorption efficiency of the anion-exchange resin, experiments to measure the reduction of phage concentration in the water sample due to resin retention were performed as follows: 0.5 g of Amberlite IRA-900 anion exchange resin (Polysciences Inc., Warrington, PA) was added to each 50 ml water samples spiked with a final concentration of 10^{5} pfu/ml of each F-RNA coliphage. Samples were incubated for 90 min at room temperature in polypropylene conical tubes with gentle mixing using a rotating sample mixer (Dynal Biotech. Inc.Lake Success, NY) set at 36 rpm. Water samples for RNA isolation (140 µl) and for plaque assay (100 µl) were taken at 0, 30, 60 and 90 min. At the end of the incubation period (90 min), resin was collected for direct RNA isolation of the adsorbed coliphages. To assess coliphage stability during the process, another 50 ml sample of water with the same coliphage concentration was incubated under the same conditions but with no resin and processed as above.

To measure the sensitivity of the resin-based concentration method, 50 ml water samples inoculated with different concentrations of coliphages, ranging from 10^{-1} to 10^{2}

pfu/ml were incubated with 0.5 g resin as described above. RNA isolation was performed on water samples at 0 min and 90 min.

4.2.3 RNA isolation

For isolation of coliphage RNA from water samples, 140 µl of each sample was processed using the QIAmp viral RNA kit© (Qiagen, Valencia, CA) according to the manufacturer's instructions. For direct RNA isolation of the coliphages adsorbed onto the resin, the resin was allowed to settle for one minute, and water was decanted. Excess liquid was removed using a pipet tip, and 560 µl of AVL buffer (from the QIAmp kit) was added to the resin. After a10 min incubation step with occasional agitation, the supernatants were transferred to 1.5 ml Eppendorf tubes and RNA isolation was performed according to manufacturer's instructions. For both water and resin samples, the RNA was eluted in 60 µl of AVE buffer (from the QIAmp kit).

4.2.4 Nucleic acid detection

Real time reverse transcription PCR (RT-PCR) reactions to detect the four different genogroups of coliphages were performed in a StepOne Plus thermocycler (Applied Biosystems, Foster City, CA) using the OneStep RT-PCR kit (Qiagen). For MS2, GA and HB-P22 (coliphage genogroups I, II and IV), detection was achieved as described previously (Pérez-Méndez et al., 2013) with primers and probes (Integrated DNA Technologies Inc., Coralville, IA) designed by Friedman et al. (2011). Briefly, real

time RT-PCR reactions (15 µl) contained 5 µl of purified RNA, 0.6 µl of enzyme mix, 3 µl of 5× buffer, 0.4 mM (each) deoxynucleotide triphosphates, 1.2 U RNAse inhibitor (Qiagen) and 300 nM of hydrolysis probes labeled at the 5´end with 6-FAM and at the 3´end with Iowa Black FQ quencher. The primer concentration of each forward and reverse primer was 600 nM for MS2 and 800 nM for GA and HB-P22. Thermocycling conditions were: 30 min at 50°C, 15 min at 95°C, 40 cycles of 1 min at 95°C, 30 sec at 60°C and 1 min at 72°C. Coliphage Qβ (genogroup III) was detected using a different system from the one described by Friedman et al. In our laboratory, primers designed by Kirs and Smith (2007) resulted in a more sensitive Qβ detection when a modified real time RT-PCR assay was used. Reaction and thermocycling conditions were as described above, without the use of a probe, 100 nM of each forward and reverse primer were used and 0.3 µl of 10× SYBR Green solution (Sigma-Aldrich) was added. A melting curve analysis was included at the end of the real time RT-PCR assay, and only positive reactions with amplicons with the same melting temperature as the Qβ coliphage stock (84.6°C) were considered positive.

Quantitative standard curves for each assay were generated using ten-fold serial dilutions of F-RNA coliphage stocks. RNA was isolated from three independent replicates of each coliphage dilution (10^{-1} to 106 pfu/140 µl, as determined by plaque assay) and 5 µl of obtained RNA was assayed by real time RT-PCR assay. Cycle thresholds (Ct) were plotted against the logarithm of pfu/reaction using StatPlus:mac LE 2009 to conduct linear regression analysis to determine the correlation coefficient (R^2) and slope. Amplification efficiency (E) of the reaction was calculated using the formula $E = 10^{(-1/m)} - 1$, where m = slope of the regression lines of the standard curves. The

limit of detection of the real time RT-PCR reaction was considered to be the minimum number of plaque forming units (pfu) detected as positive in all three replicates.

4.2.5 Statistical analyses and interpretation of sensitivity

Statistical analyses were performed using StatPlus LE.2009. F-RNA coliphage titer or threshold cycle means at different adsorption times were compared to time 0 using a Student´s t-test. Adsorption efficiency at 90 min (A90) was determined indirectly by subtracting the residual coliphage concentration found in the water sample after 90 min adsorption from the starting coliphage concentration of the sample according to the equation: $A_t = [(T0– T90)/T0] \times 100$, where T0 is the coliphage titer of the water at time 0 of incubation and T90 is the coliphage titer of the water after 90 min of incubation with the resin (residual titer). A_{90} for the different coliphages were compared using a one way ANOVA test. If significant ($p < 0.01$), Tukey's HSD test was used to identify the means that were significantly different ($p < 0.01$) from each other. Since there was no elution of virus particles from the resin following adsorption, the percentage of recovery was calculated based on real time RT-PCR results, transforming the obtained Ct into target copies using the parameters defined in the quantitative standard curves. The percentage of recovery was calculated using the equation: (total recovered copies/total inoculated copies) × 100. The increased sensitivity associated with resin use was determined using change in Ct (ΔCt). In real time RT-PCR, the Ct value decreases with an increasing amount of template in the initial sample, and theoretically, in a 100% efficient reaction, one PCR cycle gained represents twice as

much target in the initial sample. Therefore, an increase in sensitivity was calculated as 2ΔCt. The sensitivity of the concentration method was considered to be the lowest number of pfu/ml that could be detected in all three replicates using the resin.

4.3 Results

4.3.1 Standard curves

Table 4.1 summarizes the characteristics of the real time RT-PCR standard curves. Efficiencies of the real time RT-PCR reactions ranged from 95.99% to 106.65%, and all R^2 were above 0.976. Reactions were linear over a span of 6 logs (10^0 to 10^5 pfu/reaction). The limit of detection of the real time RT-PCR reactions (measured as the lowest quantity of F-RNA coliphage detected as positive in all three replicates) was 0.120 pfu/reaction for MS2, 0.345 pfu/reaction for GA, 0.013 pfu/reaction for Qβ, and 0.203 pfu/reaction for HB-P22.

Table 4.1 Characteristics of standard curves for the real time RT-PCR assays[a].

Subgroup	Slope	Intercept	Efficiency	R^2	Limit of detection[b] (pfu in 5µl of RNA)
I	-3.316	33.097	100.23%	0.988	0.12
II	-3.422	30.864	95.99%	0.976	0.345
III	-3.17	30.91	106.65%	0.986	0.0132
IV	-3.463	29.94	94.42%	0.979	0.203

[a] Standard curves were performed in three independent replicates.
[b] The limit of detection was expressed as the lowest pfu detected as positive in all three replicates.

4.3.2 Anion exchange resin adsorption efficiency and percentage of recovery of F-RNA coliphages

To evaluate the adsorption efficiency of the anion exchange resin, 50 ml water samples inoculated with 10^5 pfu/ml of F-RNA coliphages were incubated with the resin. Coliphages remaining in the water samples were quantified using plaque assays after 0, 30, 60 and 90 min of incubation. Reduction of all four F-RNA coliphages in the water samples was evident and statistically significant ($p < 0.01$) after 30 min of incubation with the resin (Figure 4.1). After 90 min of incubation, reduction of coliphage titer in the water samples was greater than 2 logs for MS2 and approximately 2 logs for GA, Qβ and HB-P22. Adsorption efficiency at 90 min (A_{90}) was greater than 96% for all coliphages (99.74% for MS2, 96.72% for GA, 97.93% for Qβ and 97.15% for HB-P22). The difference in adsorption efficiency at 90 min was superior and statistically significant for MS2 compared to GA and HB-P22, but not compared to Qβ. There was no significant difference between adsorption efficiencies of GA, Qβ and HB-P22.

RNA isolated from the water samples or directly from the resin used in these samples was analyzed by real time RT-PCR. The Cts of RNAs collected from the resin samples were significantly lower ($p < 0.0001$) compared to the corresponding water samples, enabling earlier detection of the target by more than 5 cycles for MS2/HB-P22 and by more than 7 cycles for GA/Qβ (Table 4.2). This translates to an increase of sensitivity ranging between 51× to 212×.

Based on the number of target copies estimated using the Cts determined during the F-RNA coliphage adsorption experiments described above, and the standard curve

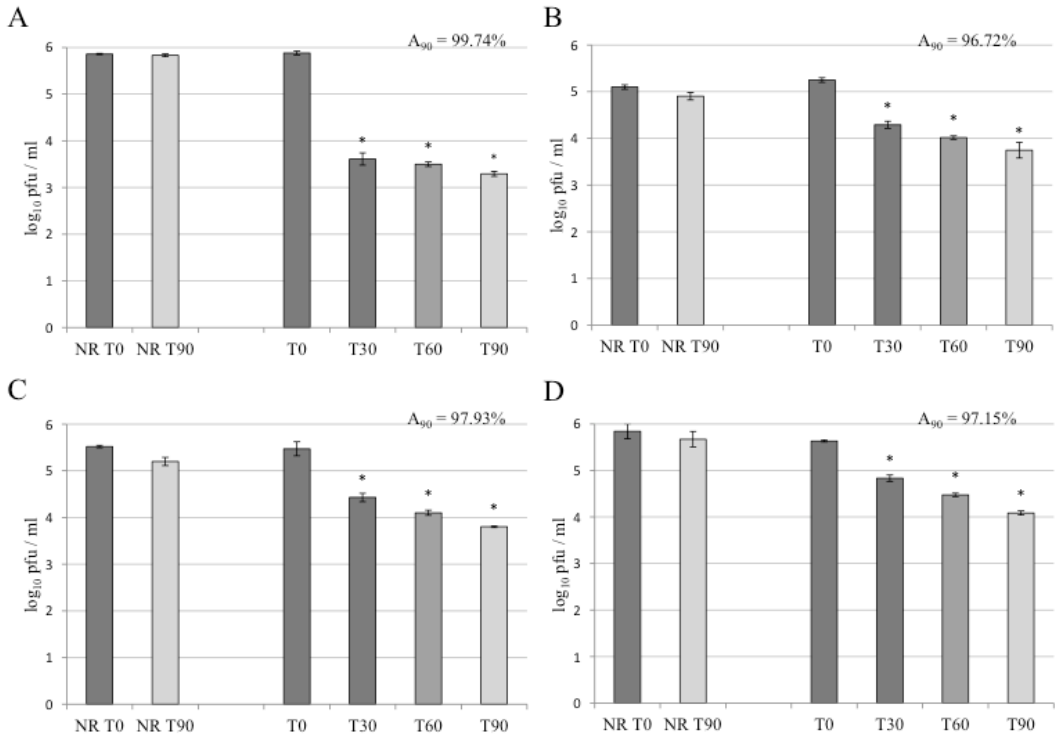

Fig. 4.1 Resin adsorption of F-RNA coliphages determined by residual titer of the inoculated water sample after incubation with the resin. Control experiment with no resin added: F-RNA coliphage titer at time 0 (NR T0) and after 90 minutes incubation (NR T90). F-RNA coliphage titer before resin addition (T0) and after 30 min (T30), 60 min (T60), and 90 min (T90) of incubation with the resin. Each bar represents titer average of three replicates. (*) p-value < 0.01 for paired t-test of T0 vs. different incubation times. T90 bars with same lower case index on them represent no significant difference (p>0.01) according to Tukey's HSD analysis. Panel A, coliphage MS2; panel B, coliphage GA; panel C, coliphage Qβ; and panel D, coliphage HB-P22. Adsorption efficiency (A_{90}) after 90 minutes incubation was calculated as described in section 2.5.

parameters, it was possible to calculate the total amount of F-RNA coliphage copies in the 50 ml water sample and the total amount of target copies adsorbed by the resin. The calculated percentage of recovery for each coliphage after a 90 min incubation was 14.46% for MS2, 34.89% for GA, 77.13% for Qβ and 12.56% for HB-P22 (Table 4.2). RNA isolated from resin incubated for 90 min in sterile water yielded real time RT-PCR negative results.

Table 4.2 Real time RT-PCR detection of F-RNA coliphages from adsorption experiments.

FRNA coliphage		Ct results (5µl of RNA)[a]			Calculated target copies in the whole sample[b]		
		Water T0	Resin	Increased sensitivity[c]	Water T0	Resin	Percentage of recovery[d]
MS2	Average	26.29	20.61*		4.83×10^5	6.99×10^4	
	Δ Ct	-	5.68	51×			14.46%
GA	Average	22.54	15.37*		1.16×10^6	4.04×10^5	
	Δ Ct	-	7.18	145×			34.89%
Qβ	Average	25.58	17.84*		2.05×10^5	1.58×10^5	
	Δ Ct	-	7.73	212×			77.13%
HB-P22	Average	21.16	15.44*		1.47×10^6	1.84×10^5	
	Δ Ct	-	5.72	52×			12.56%

[a] 140 µl of the water sample or the resin sample was used in each experiment was used for RNA isolation. RNA was eluted in 60 µl, and 5µl was analyzed in each real time RT-PCR reaction.
[b] Total target RNA copies in the sample were extrapolated from experimental Cts and the volume of sample tested, based on the standard curve generated for Table 1.
[c,d] The increased sensitivity due to the use of resin in the system and the percentage of recovery was calculated as indicated in section 2.6.
* p-value < 0.0001 for paired t-test of water T0 vs. resin result.

4.3.3 Anion exchange resin-based method sensitivity

Tap water inoculated with different concentrations (ranging between 10^{-1} and 10^2 pfu/ml) of each F-RNA coliphage was used to test the capability of the resin to concentrate viruses at low levels. RNA isolated from water prior to resin addition (T0), from water after 90 min of incubation with the resin (T90) and from the resin after 90 min incubation (R) was analyzed by real time RT-PCR (Table 4.3). MS2 was not detected in water samples at concentrations lower than 10^2 pfu/ml; however, detection from the resin was possible for all three technical replicates at 10^0 and 10^1 pfu/ml, with an

average Ct of 37 and 35 respectively. At 10^2 pfu/ml, MS2 detection in the water samples was possible for all three replicates (average Ct of 37.1), but after 90 min of incubation with the resin, detection was achieved in only two out three replicates, with an average Ct of 39.5.

Table 4.3 Real time RT-PCR results of adsorption experiments at different F-RNA coliphage concentrations.

	10^2 pfu/ml			10^1 pfu/ml			10^0 pfu/ml			10^{-1} pfu/ml		
	T0	T90	R	T0	T90	R	T0	T90	R	T0	T90	R
MS2												
Positives[a]	3	2	3	0	0	3	0	0	3	0	0	0
Average Ct[b]	37.1	39.5	35.2	-	-	35.5	-	-	37.1	-	-	-
SD[c]	0.68	0.09	0.49	-	-	0.15	-	-	0.61	-	-	-
GA												
Positives[a]	3	3	3	3	0	3	1	0	3	0	0	2
Average Ct[b]	34.6	38.6	26.7	37.7	-	29.7	39.0	-	33.1	-	-	36.9
SD[c]	1.07	0.7	0.67	0.56	-	0.05	-	-	0.22	-	-	1.14
Qβ												
Positives[a]	3	1	3	0	0	3		0	2	0	0	0
Average Ct[b]	36.7	37.9	31.9	-	-	35.6	-	-	39.0	-	-	-
SD[c]	1.1	-	0.48	-	-	0.46	-	-	0.03	-	-	-
HB-P22												
Positives[a]	3	3	3	3	3	3	3	0	3	0	0	3
Average Ct[b]	31.2	34.7	25.7	33.7	35.4	29.1	37.1	-	32.4	-	-	36.6
SD[c]	0.29	0.36	0.15	0.37	0.29	0.4	0.35	-	0.03	-	-	0.31

[a] Number of replicates with positive result (Ct > 40)
[b] Average Ct: Average threshold cycle of three replicates
[c] Standard deviation. When only one sample or no samples were positive, standard deviation was not calculated (-).
Note: Water samples before resin addition (T0), water samples after 90 minutes adsorption (T90), or resin after 90 minutes of adsorption (R) were used for RNA isolation and subsequent real time RT-PCR.

Similarly, Qβ was not detected in water at coliphage concentrations lower than 10^2 pfu/ml, but detection from the resin was possible in two of three replicates at

concentrations of 10^0 pfu/ml and in all replicates at concentrations of 10^1 pfu/ml. For higher concentrations of Qβ (10^2 pfu/ml), detection was possible by directly testing the water; however, using the resin increased the sensitivity of real time RT-PCR detection by 4.8 cycles (Ct of 36.7 to 31.9). At concentrations of 10^1 and 10^2 pfu/ml, GA was detected in all three water sample replicates. Nonetheless, GA detection sensitivities improved using the resin-based method compared to direct water testing. GA at a concentration of 10^{-1} pfu/ml was not directly detectable in the water sample, but the use of resin allowed for detection in two out three replicates. At 10^0 pfu/ml, GA was not reliably detected through sampling of the water (one of three replicates were positive), but the use of resin enabled detection in all three replicates, with an average Ct of 33.1. Finally, at 10^1 pfu/ml, GA was detected directly from water (Ct of 37.7), and after incubation with the resin, water sample replicates tested negative as a consequence of viral adsorption by the resin. Following resin adsorption, real time RT-PCR detection was enhanced by 8 cycles (Ct of 29.7). Likewise, HB-P22 was not detected in water at concentrations of 10^{-1} pfu/ml, however, it was readily detected (average Ct of 36.6) in all three resin-processed replicates. At concentrations where HB-P22 is detected directly from the water, the use of resin increased detection by 4.7 cycles (10^0 pfu/ml), 4.6 cycles (10^1 pfu/ml), and 5.5 cycles (10^2 pfu/ml).

4.4 Discussion

Although procedures to concentrate viruses from water have improved over time, these methods still have deficiencies and limitations that need to be addressed

including variable recovery efficiencies, cumbersome usage, the requirement for expensive equipment not easily adapted to field conditions and prohibitive costs (Calgua et al., 2013; Ikner and Gerba, 2012). Of these methods, filter-based techniques are the most widely used; however, the use of filters limits the speed of the procedure, is ineffective for processing samples with high particulate matter and demands relatively large volumes of eluent for virus recovery (Ikner and Gerba, 2012; Wyn-Jones and Sellwood, 2001). In this study, we tested the use of an inexpensive anion exchange resin as a viral adsorbent in a batch format. This system, in conjunction with direct nucleic acid isolation, provided a novel alternative for viral concentration from water that overcomes common problems such filter clogging, elution steps, secondary concentration steps and introduction of PCR inhibitors to the system from viral elution buffers.

The efficiency of the IRA-900 anion exchange resin to adsorb the four F-RNA coliphages tested in this study was between 96.72% and 99.74% (Figure 4.1), and is comparable to the best results demonstrated for filter-based methods (Goyal et al., 1980; Ikner et al., 2011; Sobsey et al., 1990). For example, Goyal et al. (1980) and Sobsey et al. (1990) reported the adsorption efficiency of MS2 (at concentrations ranging between 10^4 pfu/ml and 10^8 pfu/sample) in tap water to range between 91.00% to 99.89%, and only after extensive optimization and adjustment of sample pH and magnesium concentration. In our system, no conditioning of the sample was needed. Further, the majority of viral adsorption to the resin occurred within 30 min of incubation, suggesting that a simple mixing procedure is enough to promote interaction of the resin with most viral particles in the sample.

Even though the four F-RNA coliphages used in this study are structurally similar, subtle differences in their adsorption efficiency to the resin were noted. These differences may be a result of the dissimilar pI of the four F-RNA coliphages, the pI of MS2 is 3.5, Qβ is 2.7, GA is 2.1 and SP (an ortholog of HB-P22 for which no pI data is available) is 2.1 (Michen and Graule, 2010). However, adsorption to the anion-exchange resin was not more efficient for F-RNA coliphages with lower pI (and thus a more negative charge), as expected. Adsorption of GA and HB-P22 was statistically lower ($p<0.01$) compared to MS2 adsorption, which is the F-RNA coliphage with the highest pI. Other physical properties of the virion such as hydrophobicity and aggregation propensity have been suggested to account for variability of coliphage adsorption to different materials (Gerba, 1984; Langlet et al., 2008; Lukasik et al., 2000; Zerda et al., 1985). Langlet et al. (2008) observed that F-RNA coliphages GA and SP displayed similar hydrophobicities and are more hydrophobic than Qβ and MS2, with MS2 being the least hydrophobic of all of the coliphages. Therefore, minor differences in the coliphage adsorption efficiencies determined in this study may be a function of coliphage hydrophobicity, where the least hydrophobic coliphage (MS2) adsorbed with the greatest efficiency, Qβ with intermediate efficiency, and more hydrophobic coliphages (GA and HB-P22) adsorbed with the least efficiency.

Notwithstanding the high adsorption efficiency, the increase in detection sensitivity was lower than expected. As the resin adsorbed more than 96% of the viruses present in each 50 ml sample (Figure 4.1), the theoretical maximum ΔCt for the IRA-900 resin-concentrated 140 µl sample would be at least 8.42 ($\log_2 (50/0.140) \times 0.96$). However, based on the ΔCts observed (Table 4.2), only a fraction of this

maximum was achieved for each coliphage (ΔCts of 5.68 for MS2, 7.18 for GA, 7.73 for Qβ and 5.72 HB-P22). This result is corroborated by the percentage of recovery of the target from the resin data (Table 4.2), indicating that the recovery of coliphage RNA from the resin is not 100% efficient. Less than ideal recovery efficiency may be due to entrapment of virions inside the resin, incomplete lysis of virions or due to adsorption of released RNA (which is negatively charged) onto the resin. Although the four coliphages tested here share structural similarities, the percentage of recovery of RNA target copies (Table 4.2) differed. It is possible that variations between these coliphages alter the efficiency of the lysis procedure used. For example, Qβ is less resistant to desiccation on paper or to various inactivation processes (Pérez-Méndez et al., 2013; Schaper et al., 2002), a condition that may help to explain why RNA recovery is better for this specific coliphage. Nonetheless, the overall efficiency of the resin-based method compared favorably to previous reports where between 7.1% to 93.2% efficiency is documented for MS2 in tap water (Goyal et al., 1980; Ikner et al., 2011; Logan et al., 1980; Sobsey et al., 1990).

The ultimate goal of methods to concentrate virus from water is to increase detection sensitivity when samples have low concentrations of viral particles. Resin-based concentration facilitated successful detection of the four tested F-RNA coliphages in tap water samples inoculated with 10^{-1} pfu/ml (GA and HB-P22) or 10^{0} pfu/ml (MS2 and Qβ). Moreover, this method always increased detection sensitivity compared to direct water testing (Table 4.3). The sensitivity of the resin-based method was comparable to another study where low concentrations of a F-RNA coliphage (MS2, 100 pfu/sample) were successfully detected (Sobsey et al., 1990). In the aforementioned

study, adsorption of MS2 to cellulose filters ranged from 5% to 99% efficiency, depending on the pH and magnesium concentration used, and recovery from the filter ranged from 6.3% to 89%. Further, the resin method demonstrated high reproducibility (highest standard deviation is 1.1 cycles between technical replicates analyzed by real time RT-PCR) even when low virus concentrations were examined.

The use of exchange resins and other polymers for virus purification (Muller, 1950) and virus concentration (Johnson et al., 1967; Kelly, 1953) were evaluated in the 1950's in 1960's and produced promising results. However, such methods were significantly limited by the fact that viable viruses had to be eluted, often in large volumes (>400 ml), from the exchange resins in order to perform tissue culture. Now that molecular detection methods are available and often preferred, re-examination of resin-based adsorption strategies are warranted. The IRA-900 resin-based method described here offers an alternative to current virus concentration methods. It is simple to perform, is low cost (24 cents per 0.5 g), and is likely adaptable to large sample volumes. Moreover, preliminary data in our laboratory suggests that this system is compatible with a wide array of water types.

Chapter 5

Field Evaluation of a Resin-based Method for Concentration and Detection of F-RNA Coliphages from Different Water Sources

5.1 Introduction

The microbiological safety of drinking water is one of the most important tools to prevent diarrheal diseases globally. Contaminated water has the potential to cause illness to great numbers of people in countries at all levels of economic development (WHO-OECD, 2003). Fecal contamination of water is the most important source of microbiological hazard due to the probable content of pathogenic bacteria, parasites and viruses (Figueras and Borrego, 2010). An individual may be exposed to such contamination through a variety of sources such as drinking water, recreational water, irrigation and food processing water or seafood produced in impacted environments (Bosch et al., 2008). Moreover, fecally polluted water is not only a risk for human health, it also results in significant economical losses due to closure of recreational beaches and shellfish harvesting areas (Rabinovici et al., 2004).

Public health officials, aware of the critical role of fecal contamination in pathogens transmission, directed their efforts to detect water fecal contamination, and this emphasis began the practice of utilizing bacterial indicator organisms to monitor water quality (Leclerc et al., 2000; WHO-OECD, 2003).

Enteric viruses, a major cause of diverse waterborne diseases, are dispersed by shedding in very high numbers in stools and are highly infective, requiring the fewest number to cause infection of all food and waterborne microorganisms (Reynolds et al., 2008; Richards, 2001). Most waterborne enteric viruses survive extremely well in the environment and are more resistant to heat, disinfection and pH changes than most bacteria (Koopmans and Duizer, 2004). In consequence, interventions designed to decrease the content of bacterial pathogens and indicators in treated water are frequently insufficient to ensure viral quality of the water. Additionally, this differential behavior of viruses in the environment makes unsafe to rely on bacterial indicators to assess the virological quality of any kind of water (Bosch, 1998; Sobsey et al., 1990), stressing the need for viral indicators of fecal contamination and viral surrogates.

F-RNA coliphages constitute the family *Leviviridae* of icosahedral capsid, single stranded positive sense RNA bacteriophages (King, 2012). F-RNA coliphages share important characteristics with human pathogenic enteric viruses: they proliferate in the gastrointestinal tract of warm-blooded animals, are shed exclusively in feces and display similar chemical/environmental sensitivities (Grabow, 2001; Havelaar et al., 1993a). These factors have contributed to the recognition of F-RNA coliphages as one of the three groups of microorganisms useful as fecal indicators (EPA, 2000), useful as indicators of enteric viruses in shellfish and fresh water (Doré et al., 2000; Havelaar et al., 1993) and useful surrogates for human enteric viruses in studies of viral persistence in surface water and wastewater disinfection (Bae and Schwab, 2008; Tree et al., 2005).

The family *Leviviridae* of F-RNA coliphages comprises two genus: *Levivirius* and *Allolevivirus.* Based on genetic and serological properties, genus *Levivirus* is further classified in two genogroups: genogroup I (GI) and genogroup II (GII). Similarly, genus *Allolevivirus* is classified in genogroup III (GIII) and genogroup IV (GIV). It has been reported that different genogroups have different preference for particular hosts: GI and GIV have been found predominantly associated with animal feces and genogroups II and III are abundant in human sources of fecal contamination (Osawa et al., 1981; Schaper et al., 2002b). This association provides very useful information for tracking the origin of fecal pollution: the presence of particular genogroups in a given water sample can be used to identify the host or environment that is impacting the water tested. Identifying dominant sources of fecal contamination is critical for accurate assessment of public health risks and implementation of proper interventions and best management practices (Wong et al., 2012). Although some reports indicate that F-RNA coliphage genogroup/host associations are not absolute (Jofre et al., 2011; Noble et al., 2003; Scott et al., 2002), it has been demonstrated that it is possible to differentiate fecal origin from human and non-human sources using principle coordinate analysis of the genotype presence information (Lee et al., 2011; Wong et al., 2012).

Viral pathogens and F-RNA coliphages are present in water environments at low concentration, thus sensitive techniques for detection and virus concentration procedures are frequently needed to facilitate their identification in the environment. Virus concentration methods have been widely developed, and currently the most commonly used systems take advantage of the surface electric charge of the viral capsid. In these systems, the water sample is forced through membrane or cartridge

filters electrostatically charged. Viruses interact with the filter and adsorb to their surface. Further elution of the viruses from the filters using small volumes of high-protein content buffers or extreme pH facilitates concentration. Adsorption-elution based virus concentration methods are successful for some matrixes or viruses but not for others, and currently, there is no virus concentration method with adequate sensitivities, applicable to a wide range of viruses and water matrixes that offers a cost-effective and technically simple solution for continuous monitoring of water (Ikner and Gerba, 2012; Lambertini et al., 2008; Victoria et al., 2009).

In this study, we conducted a field-evaluation of a novel virus concentration method based on the use of an anionic exchange resin as an alternative to electropositive filters. In this method, the resin is dispersed in the water sample to facilitate adsorption of negatively charged F-RNA coliphages, followed by direct isolation of nucleic acids and real time RT-PCR detection. The evaluation was performed using a wide variety of water samples suspected of fecal contamination. Each sample was analyzed for detection of naturally present F-RNA coliphages and, in order to provide information about the inhibitory factors present in the water samples, a duplicate of each sample was spiked with a known amount of F-RNA coliphages providing a positive control for each of the water samples tested. The resin-based method successfully detected the F-RNA coliphages in most of the spiked samples and facilitated the detection of F-RNA coliphages naturally present in the water samples.

5.2 Materials and methods

5.2.1 F-RNA Coliphages and bacterial strains

Bacterial host *E. coli* HS (pFamp)R (ATCC 700891) and F-RNA coliphage genogroup I (MS2 ATCC 15597-B1) and genogroup III (Qβ ATCC 23631-B1) were obtained from the American Type Culture Collection (Manassas, VA). F-RNA coliphage GA (genogroup II), MX-1 (genogroup III) and BR-1 (genogroup IV) were kindly provided by Stephanie Friedman (US EPA, Gulf Breeze, FL, USA). Host bacteria and coliphage stocks were prepared as described in the US EPA method 1602 (EPA, 2001), using tryptic soy broth (TSB) (Difco laboratories, Detroit, MI) supplemented with 5 mM magnesium chloride and 50 µg/ml of ampicillin and streptomycin (Sigma-Aldrich, Saint Louis, MO). High titer F-RNA coliphage stocks were filtered through a 0.22 µm low protein-binding filter (PALL Life Sciences, Ann Arbor, MI) and stored at -70 C in 20% glycerol. The phages were enumerated using the double agar overlay plaque assay (Hershey et al., 1943) as modified by Kropinski et al. (2008) using 1.5% tryptic soy agar in the bottom layer and 0.7% tryptic soy agar in the top layer.

5.2.2 Water samples

In September 2012, a total of 65 water samples (4L each) were collected from different sites of the great Boston area in 5 sampling days. Samples included storm water outfall (n=21), brook (n=24), canal (n=2), drain (n=3), river (n=8), storm drain (n=5) and tidal creek (n=2), for a total of 50 samples of fresh water and 15 samples of marine water (N=65). Selected sampling sites were suspected of some amount of

mainly human fecal impact. Twelve sites were visited in three consecutive days. Overall, 39 different sites were sampled. Samples were collected in polypropylene sterile bottles and 50 mg/L of sodium thiosulphate pentahydrate (Mallinckrodt Baker Inc. Phillipsburg, NJ) was added to each container for dechlorination. Immediately after collection, samples were placed in coolers and transported to the lab. Upon arrival, a 50 ml aliquot of each water sample was collected in a polypropylene conical tube and stored at -80C for further total suspended solids (TSS), total dissolved solids (TDS), total organic carbon (TOC) and pH analysis. The remaining samples were refrigerated for processing for virus concentration within the next 12 hours.

5.2.3 Spiked water samples and spiked buffer controls

From each sample, two aliquots of 1 L each were transferred to 1L sterile polypropylene bottles. One of the 1L samples (spiked sample) was spiked with a coliphage cocktail containing 10^6 pfu of each F-RNA coliphage: MS2 (GI), GA (GII), Qβ and MX1 (GIII), and BR1 (GIV) and thoroughly mixed. Both spiked and unspiked samples were kept at 4C until further processing for viral concentration and RNA isolation within the following 2 hours. Additionally, a spiked control (S-PBS) was included on each sampling day, consisting of 1L of 1:4 diluted PBS, pH 7.4 spiked with the same amount of F-RNA coliphage cocktail as the spiked duplicates. To avoid cross contamination, unspiked samples were processed for viral concentration and RNA isolation prior to processing spiked samples each day.

5.2.4 Virus concentration method

Prior to virus concentration, water samples were clarified by filtration through a DIF-IN30 disposable inline filter (United filtration, Sterling Heights, MI) and transferred to sterile glass bottles using a peristaltic pump. Amberlite IRA-900 anion exchange resin (Polysciences Inc., Warrington, PA) was added to each spiked and unspiked samples (0.5 g/L) and incubated for 120 min at room temperature with continuous mixing using a stir bar and stir plate. At the end of the incubation period, the resin was allowed to settle for approximately one minute, most of the water was discarded, and resin and remaining water (approx. 30ml) was transferred to a 50 ml conical tube. The resin was allowed to settle and liquid decanted from the tube. The resin was immediately processed for RNA isolation

5.2.5 RNA isolation

Nucleic acid isolation was performed directly from the F-RNA coliphages adsorbed to the resin, with no previous elution of the viruses. Excess liquid was removed from the resin using a pipet tip, and 560 µl of AVL buffer [QIAmp viral RNA kit© (Qiagen, Valencia, CA)] was added. After 10 min incubation with occasional agitation, the supernatant containing released RNA was transferred to 1.5 ml Eppendorf tubes and RNA isolation was performed according to the kit manufacturer's instructions. To maximize RNA yield, the final sample of RNA was eluted from the column in two steps, each one consisting of incubation of the column for 5 minutes with 40 µl of AVE buffer,

followed by centrifugation at 6,000 ×g for 1 minute. Both eluates were mixed for a final volume of 80 µl of concentrated RNA.

5.2.6 Real time RT-PCR assays

Real time reverse transcription PCR (RT-PCR) reactions for detection of F-RNA coliphage GI, GII and GIV were performed in a CFX96 real time PCR detection system (Bio-Rad laboratories, Hercules, CA), with primers and hydrolysis probes (Tib Molbiol, Adelphia, NJ) designed by Friedman et al. (2011). Probes were labeled at the 5´end with 6-FAM and at the 3´end with BBQ®. The one step RT-PCR reaction was conducted in a 15 µl volume, using the QuantiTect Probe RT-PCR Kit (Qiagen) containing 1U of RNase inhibitor SUPERase-in (Life Technologies Corporation, Carlsbad, CA) and 5 µl of sample RNA template. Primer concentration for GI was 600nM each forward and reverse primer; 1600nM for GII; and 800 nM for GIV. For all tree genogroups, the probe concentration used was 266 nM. Additionally, the internal amplification control (IAC) described by Friedman et al. (2011) was included in each reaction [(200nM of each IAC forward and reverse primer, 100nM of IAC detection probe labeled at the 5' end with Cy5 and at the 3' end with BBQ®, and 0.3 ng of IAC RNA). For full description of primers and probe refer to Nordstrom et al. (2007)]. Thermocycling conditions were: 30 min at 50°C, 15 min at 95°C and 40 cycles of 1 min at 95°C, 30 sec at 60°C and 1 min at 72°C. For detection of GIII F-RNA coliphages, a different real time RT-PCR assay was used since in our lab this system resulted in more sensitive detection of F-RNA coliphage Qβ. The reaction was performed in a StepOne

plus real time PCR system (Applied Biosystems, Foster City, CA) using the OneStep RT-PCR kit (Qiagen). Primers were designed by Kirs and Smith (2007) and amplicon was detected using SYBR Green. Briefly, real time RT-PCR reactions (15 µl) contained 5 µl of sample RNA template, 0.6 µl of enzyme mix, 3 µl of 5× buffer, 0.4 mM (each) deoxynucleotide triphosphates, 1.2 U RNAse inhibitor (Qiagen), 100 nM of each forward and reverse primer and 0.3 µl of 10× SYBR Green solution (Sigma-Aldrich). Thermocycling conditions were the same as described above and a melting curve analysis was included at the end of the real time RT-PCR assay. Only positive reactions with amplicons with the same melting temperature as the Qβ coliphage stock (84.6C to 85.2C) were considered positive. Standard curves for each real time RT-PCR reaction were generated using ten-fold dilutions of RNA purified from known titer coliphage stocks. Five µl of each dilution was assayed by real time RT-PCR. Cycle thresholds (Ct) were plotted against the logarithm of pfu/reaction and R^2 and slope values were estimated. Amplification efficiency (E) of the reaction was calculated using the formula $E = 10^{(-1/m)} - 1$, where m = slope of the regression lines of the standard curves.

5.2.7 Data analysis.

To quantify the degree of inhibition produced by the water matrix on PCR detection, the cycle threshold (Ct) obtained for each spiked sample was compared to the Ct obtained for the spiked control (S-PBS). This shift in Ct was referred to as inhibition delta Ct (iΔCt) and was calculated according to the formula iΔCt = Ct of spiked

sample – Ct of S-PBS. Inhibition was rated as inhibition 1 when iΔCt was ≤ 3.32 cycles; inhibition 2 when iΔCt was between 3.33 and 6.64 cycles; inhibition 3 when iΔCt was between 6.65 and 9.96 cycles, and inhibition 4 when iΔCt was ≥ 9.96 cycles. These cutoffs (3.32, 6.64, 9,96) were selected to facilitate a quick interpretation of the inhibitory activity of the sample: in an ideal PCR reaction, a shift of 3.32 cycles in Cts would be obtained when a 10 time difference in target concentration (one log) is found in the sample. An iΔCt of 6.64 cycles would indicate a 2 log decrease in concentration of the target, and an iΔCt of 9.96 would indicate a 3 log decrease of the target in the sample. To investigate if there was a correlation between the chemical parameters measured (TSS, TDS, TOC and pH) and the observed degree of inhibition of the real time RT-PCR assay (iΔCt), Pearson product-moment correlation coefficient (Pearson's r) was calculated for each parameter and each genogroup assay. A Fisher exact test was performed to determine if the inhibition produced on detection of each genogroup was affected by the water origin (fresh water or seawater); and to determine if the proportion of positive and negative naturally contaminated samples was different in fresh water compared to seawater. Statistical analyses were performed using StatPlus LE.2009.

5.3 Results

5.3.1 Standard curves and internal amplification controls

Efficiencies of the RT-PCR reactions were 93.5%, 96.6%, 85.5% and 89.7% for genogroups I, II, III and IV respectively. R^2 values were 0.994 or above for all reactions.

Performance of the internal amplification controls was tested for each genogroup reaction and produced sigmoidal amplification curves with average Cts of 29.2 for GI, 25.4 for GII and 27.6 for GIV reactions. As GIII real time RT PCR reaction was not probe-based, there an IAC was not included.

5.3.2 TSS, TDS, TOC and pH of the water samples

TSS, TDS and TOC analysis of the samples was performed in the Soil, Water & Plant Testing Laboratory at Colorado State University (Fort Collins, CO). Averages, standard deviations, minimum values and maximum values for each parameter are reported in Table 5.1. A possible correlation between each of these parameters and the degree of real time RT-PCR inhibition observed in the spiked samples was investigated. Pearson's r was calculated for each F-RNA coliphage genogroup reaction. None of these parameters showed a significant correlation (above 0.7) with the real time RT-PCR inhibition observed for any of the four assays. The strongest correlations found were 0.532 for TDS and 0.420 for TOC, both with F-RNA coliphage genogroup II real time RT-PCR detection.

Table 5.1 Characteristics of the water samples and their correlation to PCR inhibition.

					Person's r correlation to PCR inhibition[a]			
Parameter	Mean	Standard deviation	Min value	Max value	GI	GII	GIII	GIV
pH	7.61	0.26	7.11	8.36	-0.07	-0.16	-0.06	-0.01
TSS mg/L	1.48	6.49	0	50	-0.24	-0.14	-0.18	-0.22
TDS mg/L	7,104	8,319	51	22120	0.22	0.53	0.28	0.20
TOC mg/L	668	808	11	2513	0.20	0.42	0.25	0.18

[a] Calculated for the correlation between the chemical parameter (pH, TSS etc) and the degree of RT-PCR inhibition (iΔCt) on each genogroup reaction.

5.3.3 Detection of spiked F-RNA coliphages in water samples

5.3.3.1 Inhibition during detection of the different F-RNA genogroups.

To determine the impact of the water matrixes on the concentration and detection of F-RNA coliphages, a parallel sub-sample of each water sample was spiked with a known amount of an F-RNA coliphage cocktail and subjected to the same concentration and detection techniques as the unspiked environmental water sample. Additionally, for each sampling day, a buffer solution sample spiked with the same F-RNA coliphage cocktail (S-PBS) was processed simultaneously, to represent the optimal conditions (no inhibitors present) for virus concentration and detection. The four genogroups of spiked coliphages were readily detected in S-PBS of all sampling days, producing average Cts of 22.6 for genogroup I, 27.9 for genogroup II, 16.7 for genogroup III, and 15.0 for genogroup IV. In contrast, coliphages spiked in water samples were not detected in every one of the samples; detection of spiked coliphage genogroups I, II, III and IV was

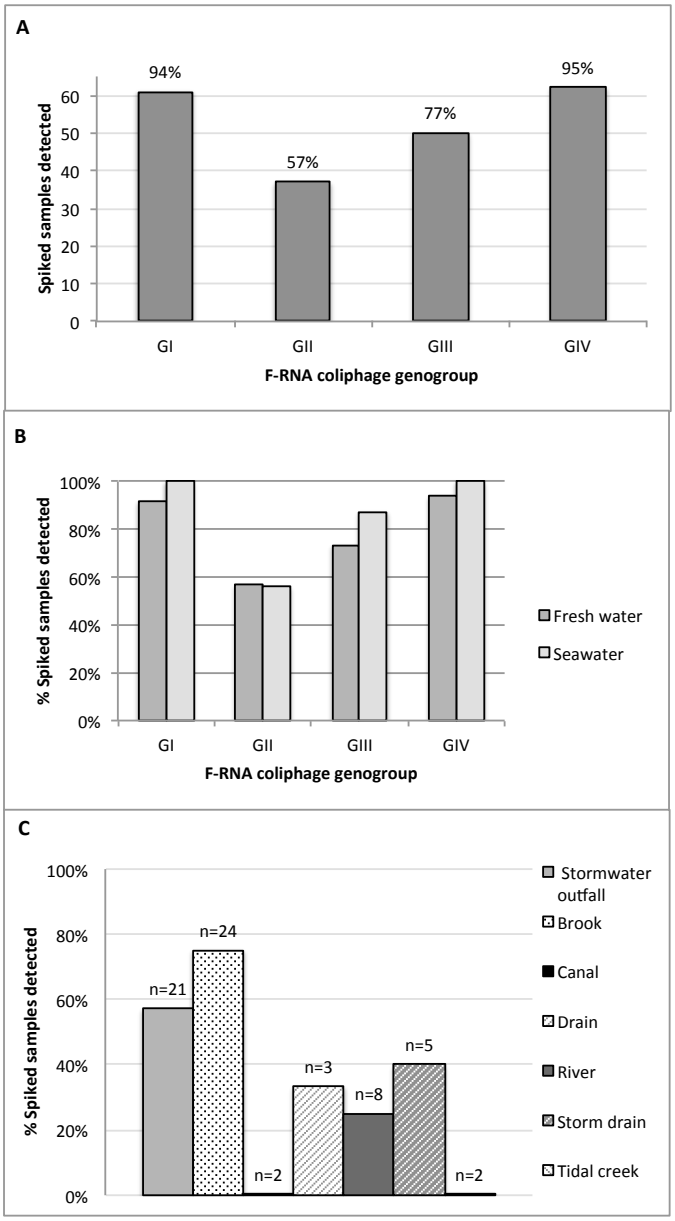

Fig. 5.1 Real time RT-PCR detection of water samples spiked with F-RNA coliphages. Panel A, samples detected as positive for each F-RNA coliphage genogroup. Panel B, percentage of spiked samples detected as positive in fresh water samples (n = 59) or seawater samples (n = 16). Panel C: percentage of spiked samples positive for all four coliphage genogroups, grouped by type of water.

possible in 94%, 57%, 77% and 98% of the samples (Figure 5.1, panel A), indicating that components of the water samples interfered differently with concentration and/or detection of each coliphage genogroup.

Table 5.2 shows detection results of the four different spiked coliphages in individual samples. For some samples (83S, 89S and 90S) detection of the four F-RNA coliphages was inhibited, but for the great majority of the samples, inhibition was selective for only one or two genogroups. When detection of only one genogroup was inhibited, it was always GII (with exception of sample S51) that was inhibited

Table 5.2 Real time RT-PCR detection of F-RNA coliphages in spiked samples[a]

Sample	GI	GII	GIII	GIV	Sample	GI	GII	GIII	GIV	Sample	GI	GII	GIII	GIV
22S	+			+	45S	+		+	+	70S	+	+	+	+
23S	+			+	47S	+		+	+	71S	+	+	+	+
24S	+			+	48S	+	+	+	+	72S	+	+	+	+
25S	+			+	49S	+	+	+	+	73S	+	+	+	+
26S	+		+	+	50S	+		+	+	74S	+			+
27S	+		+	+	51S	+	+		+	75S	+	+	+	+
28S	+		+	+	52S	+	+	+	+	76S	+	+	+	+
29S	+		+	+	53S	+		+	+	78S	+			+
30S	+		+	+	54S	+	+	+	+	79S	+	+	+	+
32S	+	+	+	+	55S	+	+	+	+	80S	+	+	+	+
33S	+			+	56S	+	+	+	+	81S	+		+	+
34S			+	+	57S	+			+	82S	+	+	+	+
35S	+		+	+	58S	+	+	+	+	83S				
36S	+	+	+	+	59S	+	+	+	+	84S	+	+	+	+
37S	+	+	+	+	60S	+	+	+	+	85S	+	+	+	+
38S	+	+	+	+	61S	+	+	+	+	86S	+	+	+	+
39S	+			+	63S	+		+	+	87S	+	+	+	+
40S	+			+	65S	+	+	+	+	88S	+	+	+	+
41S	+	+	+	+	66S	+	+		+	89S				
42S	+	+	+	+	67S	+	+	+	+	90S				
43S	+	+	+	+	68S	+	+	+	+	91S	+	+	+	+
44S	+		+	+	69S	+	+	+	+		+	+	+	+

[a] Cells in gray indicate inhibition of the spiked coliphage detection.

(27/65 samples). When detection of a second coliphage genogroup was inhibited in the same sample, this was always GIII (11/65 samples), with exception of sample 34S where the second genogroup not detected was genogroup I.

Although detection of spiked coliphages was possible in most of the samples, the level of detection varied largely. Sample's Ct was always larger compared to the Ct of the buffer spiked control (S-PBS), a positive control where optimal conditions for concentration and detection are expected. This shift in Ct (iΔCt) caused by the water matrix varied from less than one cycle to more than 10 cycles. Inhibition was rated in four levels, with inhibition 1 being the lowest (iΔCt equal or less than 3.2 cycles, which in an ideal PCR reaction would represent up to one log reduction of the target) and inhibition 4 the highest level of inhibition, with iΔCt greater than 9.96 cycles (which in an ideal PCR reaction would represent more than three logs reduction of the target). For genogroup I, 34% of the spiked samples had an inhibitory effect on the system that lowered detection by up to one log. This same level of inhibitory effect was present in 59% of the samples when tested for GII, 16% when tested for GIII and 39% when tested for GIV. The maximum level of inhibition was found in 13% of the samples when tested for GI, 7% when tested for GII, 22% when tested for GIII and 6% when tested for GIV (Figure 5.2).

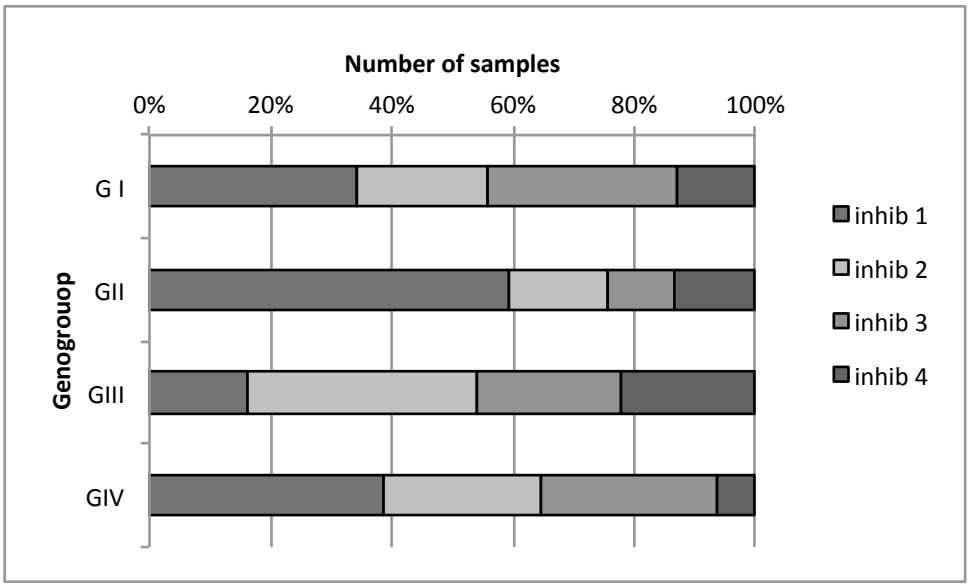

Fig. 5.2 Percentage of positive samples showing different degree of inhibition on detection of spiked F-RNA coliphages. RT-PCR result of each positive sample was compared to RT-PCR results of the S-PBS sample, degree of inhibition calculated, and rated 1 to 4 being 1 the lowest degree of inhibition (see section 2.7 for calculations). Each bar represents the total of positive samples for each genogroup. N = 61 for GI, 37 for GII, 50 for GIII and 62 for GIV.

5.3.3.2 Impact of the water origin and type of sample on detection of F-RNA coliphages

Out of the 65 water samples, 49 samples were fresh water and 16 samples were seawater. The impact of the water origin (fresh or seawater) on concentration and detection of each coliphage genogroup is showed in figure 5.1 panel B. Detection of GI was possible in 92% of the fresh water samples, and in 100% of the seawater samples. Detection of GII was possible in 57% of the fresh water samples and 56% of the seawater samples. For GIII, detection was possible in 73% of the fresh water samples and 88% of the seawater samples; and detection of GIV was possible in 94% and 100% of the fresh water and seawater samples respectively. Although for genogroups I, III

and IV the percentage of seawater samples where spiked coliphages were successfully detected was higher than the percentage of fresh water samples detected as positive, this difference was not statistically significant (p-value > 0.05). The percentage of samples that allowed for detection of the four genogroups of spiked coliphages varied greatly for the different types of water tested (Figure 5.1, panel C). Detection of spiked coliphages was more readily achieved in storm water outfall and brook samples, where detection of the four coliphage genogroups was possible in 57% and 75% of the samples respectively, compared to the other water types tested where 0% (canal), 33% (drain), 25% (river), 40% (storm drain) and 0% (tidal creek) of the samples allowed for detection of the four spiked coliphage genogroups.

5.3.3.3 Detection in diluted RNA samples

In order to determine if PCR inhibitors contributed to the decrease in detection of F-RNA coliphages in spiked water samples, a 1:5 dilution of the RNA sample was tested using the same PCR system. For samples not detected using the undiluted RNA sample, detection was achieved in 50%, 36%, 33% and 67% of the samples when a 1:5 dilution of RNA was tested (Table 5.3). Thus, the overall detection of spiked F-RNA coliphages was 97% for GI, 72% for GII, 85% for GIII and 98% for GIV.

Table 5.3 Real time RT-PCR detection of spiked F-RNA coliphages using RNA diluted 1:5

	GI	GII	GIII	GIV
Negative samples using undiluted RNA	4	28	15	3
Positive samples using diluted RNA[a]	2 (50%)	10 (36%)	5 (33%)	2 (67%)
Total spiked samples detected[b]	63 (97%)	47 (72%)	55 (85%)	64 (98%)

[a] Samples that turned positive when testing RNA diluted 1:5 in molecular grade water.
[b] Positives in the undiluted RNA assay plus positives in the diluted RNA assays

5.3.4 Internal amplification control

The internal amplification control included in this study was the same (in nature and concentration) for all three RT-PCR assays (GI, GII and GIV). However, the behavior of the IAC was different in each real time RT-PCR assay. For example: 7 samples that failed to amplify the IAC when a GI detection system was used, promoted amplification of the IAC when the GIV assay was used, and only one of them failed to amplify the IAC when the GII assay was used. The aforementioned samples were target-negative for all three F-RNA genogroups, then, failure to support IAC detection was not due to competition for substrate or enzyme. Likewise, none of the 4 target-negative samples that failed to support IAC detection in the GIV assay showed inhibition of IAC detection when the GI or GII system was used. In addition, inhibition of the IAC was not in agreement with inhibition during detection of the spiked coliphage: Table 5.4 shows the results of IAC detection of those samples where detection of the spiked coliphage was inhibited. For genogroup I, 75% of the samples where detection of the target was inhibited showed inhibition of the IAC also. For genogroup II, only one

sample (4%) of the spiked samples where target detection was inhibited showed IAC inhibition. For genogroup IV, none of the samples where detection of the target was inhibited showed inhibition on the IAC. Due to the nature of the genogroup III detection assay (SYBR Green), the internal amplification control was not included for GIII.

Table 5.4 Inhibition of internal amplification control detection in samples where spiked coliphages detection was inhibited

	GI	GII	GIV
Target detection inhibited	4	28	3
IAC inhibited	3 (75%)	1 (4%)	0 (0%)

5.3.5 Naturally contaminated water samples

Despite the presence of compounds that inhibited detection of the spiked coliphages, F-RNA coliphages naturally present in the samples were detected. While genogroup I F-RNA coliphage was not detected in any of the samples, 4 water samples resulted positive for GII detection, 15 samples were positive for GIII detection and 23 samples were positive when tested for GIV presence (Table 5.5).

Table 5.5 Real time RT-PCR results of naturally contaminated water samples

	GI	GII	GIII	GIV
Positive samples	0	4	15	23
Additional positive using diluted RNA	0	5	12	3
Total positive samples (n = 65)	0	9 (14%)	27 (42%)	26 (40%)
Fresh water (n = 49)	0	8 (16%)	21 (43%)	17 (35%)
Seawater (n = 16)	0	1 (6%)	6 (38%)	9 (56%)

Every sample was re-tested using a 1:5 dilution of RNA, producing additional positive results for all genogroups except genogroup I. In summary, the total number of samples where naturally present coliphages were detected was 9 for GII, 27 for GIII and 26 for GIV, accounting for 14%, 42% and 40% of the tested samples respectively (Table 5.5). Some samples became negative when RNA was diluted (1 for GII, 4 for GIII and 10 for GIV). When samples were grouped by origin (seawater and fresh water) 16% of the freshwater samples and 6% of the seawater samples resulted positive for GII. For genogroup III, 43% of the fresh water and 38% of the seawater samples tested resulted in positive reactions. Finally, 35% of the fresh water and 56% of the seawater samples resulted positive for genogroup IV F-RNA coliphage (Table 5.5). The amount of fresh water samples that tested positive was not statistically different to the amount of seawater samples found positive for any of the four F-RNA coliphages tested ($p < 0.05$).

Table 5.6 shows the real time RT-PCR detection of the four F-RNA coliphage genogroups for each of the naturally contaminated samples tested. In 25 of the

samples no coliphage was detected. Twenty samples were contaminated with only one genogroup, 18 samples were contaminated with two different genogroups and 2 samples were contaminated with 3 different F-RNA coliphage genogroups. Shadowed cells indicate failure to detect the spiked coliphage in the parallel sample (see table 5.2), suggesting that detection of naturally present F-RNA coliphages in those samples may had been hampered by the water matrix. In four samples (samples 57 and 89 for genogroup II and samples 51 and 66 for genogroup III), detection of naturally present coliphages was possible although spiked coliphages were not detected in the spiked sample. In all four samples, detection of the naturally present coliphages was achieved only when diluted RNA sample was used.

Overall, 62% of the tested samples showed some sort of fecal contamination: F-RNA coliphages of human origin were found in 46% of the samples, and F-RNA coliphages of animal origin were found in 40% of the samples (Table 5.7). In 15% of the samples it was possible to detect coliphages of both, human and animal origin.

Twelve sites were sampled on three consecutive days and results are presented in table 5.8. For some sites, presence of inhibitors for detection of F-RNA coliphages was constant throughout the three sampling days (sites 1 and 3, GII detection) while for other sites no inhibitors were present on any sampling day (sites 2, 5, 6, 7 and 9). Sites 8 and 12 were free of inhibitors on the 2 first days of sampling and inhibition for all genogroup detection was present on the third day of sampling only. In the same way, coliphage presence was constant for some samples on consecutive days (samples 2 and 7, GIV; sample 6, GIII) while intermittent for others.

Table 5.6 Real time RT-PCR detection of the different F-RNA coliphage genogroups in naturally contaminated water samples[a]

Sample	GI	GII	GIII	GIV	Sample	GI	GII	GIII	GIV	Sample	GI	GII	GIII	GIV
22					45					70				+
23					47					71			+	
24					48		+	+	+	72			+	+
25					49		+	+		73				+
26					50					74				
27			+		51			+		75		+	+	
28			+		52					76			+	+
29					53					78				
30					54			+		79		+	+	+
32					55			+		80				+
33					56			+	+	81				+
34			+		57		+			82			+	+
35				+	58		+	+		83				
36					59			+	+	84				
37			+	+	60					85			+	
38					61				+	86		+		+
39					63					87		+	+	
40					65			+	+	88				+
41			+	+	66			+	+	89		+		
42			+	+	67			+	+	90				
43				+	68			+	+	91				+
44				+	69			+	+					

[a] Cells in gray indicate PCR inhibition activity of the sample, detected by the PCR results of spiked samples (see table 2).

Table 5.7 Samples showing fecal contamination.

	Fecal contamination[a]	Human origin (GII+GIII)	Animal origin (GI + GIV)	Animal and human origin[b]
Samples	40 (62%)	30 (46%)	26 (40%)	15 (23%)

[a] Samples positive for at least one F-RNA coliphage

[b] Samples positive for at least one genogroup of human origin (GII or GIII) and at least one genogroup of animal origin (GI or GIV)

Table 5.8 Real time RT-PCR detection of the different F-RNA coliphage genogroups in naturally contaminated water samples sampled in consecutive days[a]

Site[b]	Sample[c]	GI	GII	GIII	GIV	Site[b]	Sample[c]	GI	GII	GIII	GIV
1 Stormwater outfall (seawater)	47					7 Brook (fresh water)	56			+	+
	63						73				+
	78						88				+
2 Brook (fresh water)	59			+	+	8 Brook (fresh water)	58		+	+	
	76			+	+		75		+	+	
	91				+		90				
3 Brook (fresh water)	57		+			9 Stormwater outfall (seawater)	52				
	74						69			+	+
	89		+				84				
4 River (fresh water)	53					10 Stormwater outfall (seawater)	49		+	+	
	70				+		66			+	+
	85			+			81				+
5 Brook (fresh water)	54			+		11 Stormwater outfall (fresh water)	50				
	71			+			67			+	+
	86		+		+		82			+	+
6 Brook (fresh water)	55			+		12 Drain (fresh water)	51			+	
	72			+	+		68			+	+
	87		+	+			83				

[a] Cells in gray indicate PCR inhibition activity of the sample, detected by PCR results of spiked samples (see table 2).
[b] Type of water and origin (in parenthesis)
[c] Samples within the same group of three were taken from the same site in three consecutive days.

5.4 Discussion

Despite multiple improvements to existing virus concentration methods, there is still the need for an easy, sensitive, reliable, cost effective and standardizable assay to be used in routine labs with a variety of water samples (Calgua et al., 2013; Pang et al.,

2012). The objective of this work was to investigate the performance of a recently developed resin-based methodology for concentration of F-RNA coliphages with a wide variety of water samples, to ensure the best possible field evaluation of the method. The strategy used for this evaluation was to investigate the natural presence of F-RNA coliphages in samples suspected of fecal contamination and to spike duplicates of all water samples with F-RNA coliphages to providing a positive control for each individual sample. With this approach, this study provides information for better understanding of the inhibitory characteristics of the water samples, valuable to understand the scope and limitations of the method, and for better interpretation of field studies results.

The resin-based method allowed successful detection of the spiked F-RNA coliphages in most of the samples tested (Table 5.4), and detected naturally contaminating coliphages in more than 60% of the samples (Table 5.7), with no need for costly equipment or conditioning of the samples, providing a feasible alternative to filter-based virus concentration methods.

5.4.1 Differential performance of the method for the different F-RNA coliphages

Detection of spiked coliphages GII and GIII was more frequently inhibited than detection of genogroups I and IV (Figure 5.1, panel A; table 5.2, table 5.3). The lower frequency of detection of GII and GIII may be result of several factors including lower adsorption of GII and GIII to the resin, inefficient extraction of the nucleic acids of these two coliphages, or higher susceptibility of the RT-PCR reaction used for detection of GII and GIII compared to the susceptibility to inhibitors of the GI and GIV PCR reactions.

Although results from our lab show that the resin adsorbs more than 95% of the four genogroups of F-RNA coliphages from tap water, it has been reported that diverse molecules from the water samples may interfere with the adsorption of viruses to filters (Gerba, 1984; Langlet et al., 2008; Lukasik et al., 2000) and thus, possibly interfere with adsorption of viruses to resin. Inefficient extraction of nucleic acids is not likely the cause for differential detection of GII and GIII coliphages compared to GI and GIV coliphages since previous studies in our lab indicate that the percentage of RNA recovery from GII and GIII is higher than the recovery of GI and GIV RNA (data not shown). Differential impact of inhibitors on RT-PCR reactions may be another cause for lower detection of GII and GIII. Several publications show that PCR inhibitors are concentrated simultaneously with nucleic acids (Abbaszadegan et al., 1999; Ikner and Gerba, 2012) and that these inhibitors impact differently molecular detection of different viruses (Ikner et al., 2011; Jones et al., 2009; Lambertini et al., 2008).

For source tracking purposes, the lower detection of GII and GIII may introduce a bias towards detection of animal-related fecal contamination (GI and GIV) over detection of human fecal contamination (GII and GIII), therefore, the results obtained here for the naturally contaminated samples may provide an underestimation of the human impact on the tested sources.

5.4.2 Detection of spiked coliphages in different water sources.

Salinity of the water is expected to have an impact on adsorption of viruses to charged surfaces (Gerba, 1984); however it is not clear how this factor would influence

adsorption of RNA-coliphages to the resin. It was reported that electropositive filters showed low recovery of MS2 coliphage seeded in levels of 10^6 pfu/L when NaCl was added to the water (Lukasik et al., 2000; Wong et al., 2012), but Gibbons et al (2010) found excellent adsorption of coliphage Qβ to NanoCeram cartridge in seawater inoculated with 10^{11} pfu/L. Being Amberlite IRA900 an anion exchange resin to where viruses are expected to adsorb by electrostatic charges, and given that the concentration of spiked coliphages used in this evaluation was substantially lower than the concentration used in the above-mentioned reports, the performance of the resin-based method may have been negatively impacted by seawater. However, the results obtained in this evaluation showed that the success in detection of the different spiked F-RNA coliphages (GIV > GI > GIII > GII) follows the same trend in both fresh and seawater, and no significant difference on detection of spiked F-RNA coliphages in fresh water compared to seawater (Figure 5.1, panel B) was found. On the contrary, the type of sample had a significant effect on coliphage detection (Figure 5.1, panel C) suggesting that characteristics of the water sample other than salinity have more impact on the method. It has been suggested that solids suspended in the water sample (De Keuckelaere et al., 2013; Wu et al., 2011), organic matter (Gerba, 1984; Ikner and Gerba, 2012; Logan et al., 1980; Wyn-Jones and Sellwood, 2001) and other nonviral constituents of the sample (Sobsey et al., 1990) interfere with virus concentration/detection methods. In this study, none of the physicochemical characteristics measured showed a significant association with inhibition of concentration/detection (Table 5.1), in agreement with results reported by Hill et al.

(2007) where lack of association of pH, turbidity, TOC and other water characteristics with PCR or RT-PCR inhibition was found.

5.4.3 Inhibitors on RT-PCR reactions

An internal amplification control provides a widely used tool to assess the contribution of the nucleic acid sample to inhibition of the PCR reaction: when the internal control is inhibited in a given amplification reaction, caution must be used to interpret a negative result on detection of the target, and conversely, a successful amplification of the internal control indicates that failure to detect the target was due to its absence from the sample, not due to inhibition of the PCR reaction. In this study, different behavior of the IAC in the different RT-PCR assays was found for the same RNA sample (section 5.3.4, Table 5.4), indicating that IAC performance was influenced not only by inhibitors present in the RNA sample but also by target, primers and probes specific for each assay. Additionally, when investigating the correlation of IAC performance with inhibition on detection of the spiked coliphages, two different behaviors of the IAC were noticed: a good correlation between both systems (IAC detection and spiked coliphage detection) in the GI assay, and no correlation between both systems in GII and GIV assays, where no inhibition of IAC was observed in samples where detection of the spiked coliphages was completely inhibited (Table 5.4). Although both the IAC and spiked coliphages are intended to detect the impact of the matrix sample on the tested methodology, the IAC is affected by inhibitors in the PCR reaction only, while the spiked coliphage system would detect failures at all levels of the

methodology, including adsorption of the virus to the resin, RNA isolation and RT-PCR assays. Results of table 5.4 indicate that in those samples where GII and GIV detection was inhibited, inhibition was most likely caused by interference of the water sample with coliphage adsorption or failure in RNA isolation, not due to interference with the PCR reaction (IAC was not affected). However, 36% and 67% of these negative samples were successfully detected as positive when a 1:5 dilution of the RNA sample [a strategy frequently used to detect and lower the impact of PCR inhibitors in the nucleic acid samples (Bosch et al., 2011)], was used in the RT-PCR assay (Table 5.4), demonstrating an important presence of PCR inhibitors for GII and GIV detection in the RNA samples. Failure of the IAC to accurately indicate if a PCR reaction would support amplification of the target represents an important problem for environmental virology. Regardless of the fact that internal amplification control systems are carefully designed to have no interference with target detection, and length and annealing temperatures of the used primers are designed to be compatible with the PCR system for which they are intended (Hoorfar et al., 2004), several factors may produce differential inhibition on amplification of the target or amplification of the IAC. In a study of the PCR inhibitory mechanisms of different substances (Opel et al., 2010), authors concluded that there are different modes for PCR inhibition. Some mechanisms include lowering the activity or availability of the DNA polymerase in the system, producing a homogeneous inhibitory effect for both the IAC and the target amplification. However, other mechanisms affect template availability or processivity of the polymerase in a template-specific manner, providing a suitable explanation as to how inhibitors present in an RNA sample may produce different inhibition to different primers/template systems.

This point is further supported by the fact that the degree of inhibition caused by the samples had different distribution in the different assays (Figure 5.2). It is interesting to notice that in the system with the highest failure in detection of spiked coliphages (GII), almost 60% of the positive samples showed a low level of inhibition, showing a decrease in level of detection of less than one logarithm. Although based on theoretical calculations, the estimation of inhibitory effects on the level of detection expressed as logarithms is helpful to understand why molecular techniques, recognized as very sensitive when tested in laboratory conditions with "clean" samples, fail to detect low levels of targets in field conditions. PCR inhibitors may be the cause of false negative reactions and decrease dramatically (more than 4 logs) the limit of detection of the system.

5.4.4 Naturally contaminated water samples

All sampled sites were selected because they were suspected of some amount of human fecal contamination, and most were previously identified positive for fecal indicator bacteria. F-RNA coliphages were detected in a significant percentage of the tested samples (Table 5.7) suggesting that the resin-based method performed well in the wide variety of water samples tested. Dilution of the RNA sample provided additional positive results, indicating that RT-PCR inhibitors were present in the samples. Evaluation of the method with positive controls for all the tested samples made possible a better interpretation of the results obtained with naturally contaminated samples. Genogroup I and IV were readily detected in 94% and 98% of the spiked

samples, suggesting that the frequency of detection of these genogroups in naturally contaminated samples (0% and 40% of positive samples for GI and GIV respectively, table 5.5) is a good indication of the status of the water sampled. No detection of GI in any of the samples is an unexpected result as this genogroup is frequently found in fecally impacted waters, and coliphage MS2 (GI coliphage) has been reported as the most persistent F-RNA coliphage in the environment (Schaper et al., 2002a). One possible explanation is that GI has been more associated with animal fecal contamination, and these sampling sites, mostly urban, were mainly suspected of human fecal contamination. Likewise, as detection of GII and GIII spiked coliphages was the least efficient (57 and 77% of the samples respectively), it is possible that detection of these genogroups in naturally contaminated samples was underestimated. Even so, GIII was the most frequently found genogroup in naturally contaminated samples (42% of the samples, table 5.7), in accordance with what has been previously observed for sites impacted by human fecal material (Grabow, 2001). F-RNA coliphages present in lower concentrations in the naturally contaminated samples compared to the spiked samples may be another reason for no detection of F-RNA coliphages in the water samples here tested. Although the sensitivity of the resin-based method as tested in the lab with spiked tap water is 10^0 pfu/ml for MS2 and Qβ and 10^{-1} pfu/ml for GA and HB-P22 (a GIV F-RNA coliphage) (submitted for publication), the presence of inhibitory compounds in the samples would certainly alter these limits of detection. Sampling of the same sites on consecutive days allowed the recognition of a dynamic presence of inhibitors and coliphages in those sites and supports the need for

frequent or continuous sampling in order to establish the sanitary status of a water body.

The availability of a rapid, simple and inexpensive technique for concentration and detection of F-RNA coliphages from different water sources would provide a valuable tool for environmental virology and source tracking. Results presented here and the unpredictable and continuous changes that water samples may suffer highlight the need for proper evaluation of the virus concentration method in use, in order to recognize its limitations for each sample. The evaluation strategy proposed here (introduction of a positive control for every sample tested) supplies an easy and trustable means to determine the impact of each different sample on detection of each target, which is needed for a correct interpretation of the results. Finally, the simplicity and low cost (24 cents per 0.5 of resin) of the evaluated method warrants further research to improve its performance to lower the amount of PCR inhibitors in the final sample of RNA.

Chapter 6

Concentration of Enteric Viruses from Tap Water Using an Anion Exchange Resin-based Method

6.1 Introduction

It is estimated that consumption of contaminated drinking water causes more than 19 million illnesses every year in the United States (Reynolds et al., 2008). Enteric viruses are a major cause of diverse waterborne diseases, from mild gastroenteritis to life threatening conditions such as hepatitis and meningitis. These viruses infect the intestinal tract of humans and are shed in very high numbers into the stools (Bosch, 1998; Koopmans and Duizer, 2004; Schultz et al., 2011).

Rotavirus (RV) is the most important causative agent of infantile diarrhea; it is estimated that more than 600,000 rotavirus related deaths occur worldwide each year (Parashar et al., 2006). This virus belongs to the Family Reoviridae, genus Rotavirus, and it has been detected in drinking water and linked to rotavirus outbreaks (Gratacap-Cavallier et al., 2000). Human adenoviruses (HAdV) belong to the Family Adenoviridae, genus Mastadenovirus and comprise 54 serotypes classified in seven species. The two members of the species Human adenovirus F or enteric adenoviruses (HAdV-40 and HAdV-41) are present in high amounts in feces of young children with acute gastroenteritis, and are second only to rotaviruses as a major cause of infantile viral diarrhea (King, 2012). As adenoviruses are very persistent in water, they are considered to be a conservative indicator of human viral fecal contamination (Jiang,

2006; Mena and Gerba, 2009). Hepatitis A virus (HAV) is the only species of the genus Hepatovirus, family Picornaviridae (King, 2012). HAV causes acute hepatitis in humans, replicates in the hepatocytes and is transmitted by the fecal-oral route through contaminated food and water. Worldwide, clean drinking water is an inverse predictor of HAV infection rates (Jacobsen and Koopman, 2005).

Enteric viruses have very low infectious doses, ranging from 1 to 100 viral particles (Appleton, 2000; Bresee et al., 2002; Ikner et al., 2011; Koopmans and Duizer, 2004), thus, water contaminated with very low concentrations of viral particles still represents a significant health risk. Sensitive methods to detect viruses at low concentration in water samples are needed (Wu et al., 2011) and procedures for virus concentration from water are especially critical for successful detection (Jones et al., 2009).

Although several methods for concentration of viruses from water have been developed, they are limited by the need for expensive equipment, difficulty in processing large volumes of water, low efficiency, excessive processing time, requirement for sample conditioning and incompatibility with downstream detection techniques. These problems have prompted continuous searches for new and improved concentration methodologies. At present, the most widely used virus concentration methods are based on adsorption of the viral particles to filters, taking advantage of the negatively charged surface of most enteric viruses due to their low isoelectric point. However, elution from the filters is often inefficient and leads to the use of large volumes of elution buffer that has to be reprocessed using a secondary concentration technique, lowering

the overall efficiency and increasing processing time and cost of the method (Ikner et al., 2011; Wyn-Jones and Sellwood, 2001).

The objective of this study was to test a novel and simple method to concentrate different enteric viruses from tap water. This method is based on adsorption of the viruses to an anionic exchange resin dispersed into the water sample, followed by direct isolation of nucleic acids from the resin, thus eliminating the need for elution and secondary concentration steps. Adenovirus 40 (a DNA virus), hepatitis A virus and rotavirus (both RNA viruses) were selected for this study due to their public health relevance and diversity of surface structure, size and isoelectric points (Table 6.1).

Table 6.1. Characteristics of the viruses used in this study

Pathogen	pI[a]	Nucleic acid[b]	Capsid characteristics[b]	Size[b]
Adenovirus	4.5	dsDNA	Icosahedral with protruding fibers	70-90nm
Hepatitis A	2.8	ssRNA	Icosahedral	22-30nm
Rotavirus	8.0	dsRNA	Icosahedral with trimeric spikes	80-100nm

[a] Isoelectric points. Source: Michen and Graule 2010
[b] Source: King 2010

6.2 Materials and methods

Viruses and cell lines were obtained from the American Type Culture Collection (Manassas, VA), and propagated according to their guidelines. Human adenovirus 40, strain Dugan (ATCC® VR-931TM) was propagated in HEK-293 cells (ATCC® CRL-

1573TM). Hepatitis A virus, strain HM175/18f (ATCC® VR-1402TM) was propagated in FRhK4 cells (ATCC® CRL-1688TM) and human rotavirus, strain Wa (TC adapted) (ATCC® VR-2018TM) was propagated in MA-104 clone 1 cells (ATCC® CRL-2378.1TM). The titer of the viral stocks was determined by assaying at least six replicates of 10-fold serial dilutions of the virus sample, and tissue culture infectious doses 50% (TCID50 /ml) were calculated using the Reed-Muench method (Reed and Muench, 1938).

To test the performance of the proposed concentration method, 10 L samples of dechlorinated tap water (pH = 8.2) from the Fort Collins, CO municipal water supply were spiked with each virus stock to obtain final viral concentrations of 10, 100 and 10,000 $TCID_{50}$/sample. After thoroughly mixing, a 140 µl-sample of the spiked water was used for nucleic acid isolation using the QIAmp viral RNA kit© (Qiagen, Valencia, CA) and further molecular detection of the target. For virus concentration, 0.5 g of the anion exchange resin Amberlite IRA- 900 (Polysciences Inc., Warrington, PA) was added to the 10 L spiked water sample and mixed continuously at room temperature for 90 min, using a stirring bar. All experiments were conducted in triplicate. At the end of the mixing period, stirring was stopped, the resin was allowed to settle for one minute, collected from the bottom of the 10 L glass bottle using a wide bore tip serological pipette (orifice of 2-3mm) and transferred to a 50 ml conical tube. Any remaining liquid was removed from the resin using a pipet tip, and nucleic acid isolation from the absorbed viruses was accomplished by adding 560 µl of AVL buffer (QIAmp kit) to the resin. After a 10 min incubation with occasional agitation, the supernatant was transferred to a 1.5 ml Eppendorf tube and the rest of the nucleic acid isolation was

performed according to manufacturer's instructions. For both water and resin samples, the nucleic acid (DNA or RNA) was eluted in 60 µl of AVE buffer (QIAmp kit).

All viruses were detected using commercially available real time PCR (for Adenoviruses) or real time RT-PCR (for hepatitis A virus and rotavirus) kits (Ceeram, La Chapelle-sur-Erdre, France) in a StepOne Plus thermocycler (Applied Biosystems, Foster City, CA). Five µl of nucleic acid extract from the water sample or from the resin was tested in each reaction. In addition to positive, negative and internal amplification controls provided by the kit manufacturer, nucleic acid isolated from resin added to 10L of sterile water and mixed for 90 min (negative control) was tested for all target viruses, producing negative results.

6.3 Results

HadV-40 was not detected in water samples containing 10 $TCID_{50}$, however, it was readily detected in all three replicates using the resin-based method, with an average threshold cycle (Ct) of 31.7 (Table 6.2). In addition, although water samples spiked with 100 or 10,000 $TCID_{50}$ of HAdV-40 produced positive results by real time PCR (average Ct of 36.8 and 30.3 respectively), the use of the resin-based concentration method improved target detection considerably, resulting in average Cts of 28.3 and 18.3 respectively. When the average Ct of the water samples was compared to the average Ct of the resin samples using a T-test, statistically significant differences were found for both HAdV-40 concentrations, with p-values of 0.0005 for 100 $TCID_{50}$ and 0.0003 for 10,000 $TCID_{50}$.

Table 6.2. Real time PCR and RT-PCR detection of enteric viruses in water and resin samples

		10 $TCID_{50}$/10L		100 $TCID_{50}$/10L		10,000 $TCID_{50}$/10L		
Pathogen		Water[a]	Resin[b]	Water[a]	Resin[b]	Water[a]	Resin[b]	Limit of detection[c]
Adenovirus	Average	-	31.7	36.8	28.3*	30.3	18.3*	10 $TCID_{50}$/10 L
	ΔCt[d]		ND		8.5		12.1	
Hepatitis A	Average	-	36.3	35.7[e]	33.7	35.1	30.78*	10 $TCID_{50}$/10 L
	ΔCt[d]		ND		2.0		4.3	
Rotavirus	Average	-	38.9[e]	-	36.4	37.0[e]	30.0	100 $TCID_{50}$/10 L
	ΔCt[d]		ND		ND		7.0	

[a] 140 µl of the spiked water sample was used for nucleic acid isolation and 5 µl of the nucleic acid sample was used for each real time PCR reaction.
[b] All the resin of each experiment was used for nucleic acid isolation and 5 µl of the nucleic acid sample was used each real time PCR reaction.
[c] The limit of detection was considered to be the lowest virus concentration successfully detected in all the three replicates tested.
[d] Delta Ct (ΔCt) was calculated as average Ct of the water sample minus average Ct of the resin sample.
[e] Only one out of three replicates was positive.
* p-value < 0.001 for paired t-test of water vs. resin result. This value was calculated only when all three replicates of water detection and resin detection were positive.

Similarly, HAV was not detected in the water when 10 $TCID_{50}$ were spiked in the 10 L sample, and it was detected in only one out of three replicates when spiked with 100 $TCID_{50}$. However, this virus was detected in all the replicates at both these concentrations when the resin-based method was used, producing average Cts of 36.6 and 33.7 respectively. When 10,000 $TCID_{50}$ of HAV were spiked, direct detection was possible (Ct = 35.1) but the use of the resin (Ct = 30.78) improved HAV detection significantly (p-value = 0.000007). The resin-based concentration method demonstrated similar performance when tested with RV: concentrations where direct detection from water was not possible (10 or 100 $TCID_{50}$), or possible in only one out of

three replicates (10,000$TCID_{50}$), were detected as positive using the resin-based concentration method, producing average Cts of 38.9 for 10 $TCID_{50}$ (only one replicate was positive), 36.4 for 100 $TCID_{50}$ and 30.0 for 10,000 $TCID_{50}$. The limit of detection of the concentration method for each virus was deemed to be the lowest concentration of the virus detected as positive in all three replicates. Therefore, the limit of detection of the method was 10 $TCID_{50}$/10 L (or 10^{-3} $TCID_{50}$/ml) for HAdV-40 and HAV, and 100 $TCID_{50}$/10 L (or 10^{-2} TCID50/ml) for RV.

6.4 Discussion

The shift in Ct or delta Ct (ΔCt) observed when the resin-based method was used (Table 6.2) represents an improvement in detection due to increased amount of targets in the concentrated sample. For each virus, this improvement seems to be dependent on the viral load in the sample, as it is greater for 10,000 $TCID_{50}$ than it is for 100 $TCID_{50}$. This behavior may be explained by the fact that viral adsorption to surfaces is influenced by the frequency of impacts of the virus with the adsorbent surface, and the probability of impacts increases with the virus concentration (Gerba, 1984). The improvement in detection (ΔCt) is different for the three viruses tested, being greater for HAdV-40 > RV > HAV. These different performances may be result of differential virus adsorption to the resin. Amberlite IRA-900 is a strong basic anion exchange resin that was selected as a substitute for positively charged filters, and adsorption is expected to happen through electrostatic charge interactions between the anion exchange resin and the virus. At a given pH, differences in viral pIs account for

differences in virus charge and thus for differences in adsorption. However, based on their pI (Table 1), the electrostatic charge of the viruses would be more negative for HAV > HAdV-40 > RV, a trend that does not explain the differences in improvement in detection observed for the three different viruses. Interestingly, as the pI of rotavirus (8.0, Table 6.1) is very close to the pH of tap water (8.2), this virus must have very low negative surface charge, however, the improvement in sensitivity was not the least of the three viruses tested. Thus, other factors are likely involved in the virus adsorption to the resin, such as physical entrapment of the virus to the porous resin surface (promoted by the protruding fibers of adenovirus and trimeric spikes of rotavirus) (Gibbons et al., 2010) or enhanced hydrophobic interactions as suggested for charged filters by other authors (Langlet et al., 2008; Lukasik et al., 2000).

In addition to differential adsorption to the resin, differences in nucleic acid extraction may impact the overall performance of the resin-based system. Rotavirus presented the highest limit of detection (100 $TCID_{50}$) although its ΔCt was superior to that of HAV. The rotavirus capsid is made up of three concentric protein layers (King, 2012) and it has been reported that very low yields of RNA are obtained unless additional steps such as SDS-proteinase K incubation are performed before RNA extraction (Brassard et al., 2005). Therefore, it is possible that the lower sensitivity of the concentration method for rotavirus was due to inefficient RNA isolation, and not to poorer performance of the concentration method with this particular virus. Rotavirus detection in water samples with the highest viral concentration was low (only one out of three replicates was positive), compared to detection of the two other viruses that were tested.

The majority of studies that have evaluated the performance of virus concentration methods use a high amount of spiked virus (10^5 to 10^7 viral particles / L), amounts that do not necessarily addresses the problem of detection of low concentration of viruses in water. Results of those studies, although very useful to quantify the recovery efficiencies of such methods, are not always predictors of the performance of the method in samples containing very low virus concentration, as the number of virions within the sample may impact the efficiency of recovery. Pang et al. (2012) used positively charged filters to concentrate viruses spiked into deionized water and their results suggested a trend of decreasing virus recovery as the spiked rotavirus or echovirus concentration decreases, however no such trend was detected when norovirus, adenovirus or coxsackievirus was spiked. Similarly, virus recoveries were not statistically associated with the quantity of virus seeded (poliovirus, coxackievirus, adenovirus or norovirus) in drinking or well water concentrated by glass wool filters (Lambertini et al., 2008), but both, virus type and water matrix were highly associated with virus recovery.

In this study we tested the proposed concentration method using low concentrations of three relevant enteric viruses, and demonstrated successful detection of HAdV-40 at 10 $TCID_{50}$/10L. This limit of detection for tap water spiked with adenovirus compares favorably to 3.7×10^2 $TCID_{50}$/10 L reported by Pang et al. (2012) using a NanoCeram filter method followed by flocculation as a mean for secondary virus concentration; and to 10 to 10^3 genomic copies/L obtained by Lambertini et al. (2008) using a glass wool filter and flocculation method. Likewise, by employing our method we successfully detected 10 $TCID_{50}$ of HAV virus spiked in 10L of water, comparable to

the reported limits of detection for this virus of 10 $TCID_{50}$/L using Convective Interaction Media (CIM) monolithic supports coupled to a secondary concentration process by ultracentrifugation (Kovač et al., 2009); 10^3 to 10^5 $TCID_{50}$/1.5 L using ultracentrifugation or ultrafiltration as secondary concentration method after filtration through positively charged filters (Di Pasquale et al., 2010); 45 to 3607 $TCID_{50}$/1.5 L using different filters (CIM or positively charged membrane filters) and secondary concentration methods (ultracentrifugation, ultrafiltration or direct isolation of the nucleic acid from the filter) (Schultz et al., 2011); and 0.2 to 20 $TCID_{50}$/L using a positively charged filter followed by sonication (Butot et al., 2013). For rotavirus our method showed lower sensitivity than for the other two viruses evaluated, with a detection limit of 100 $TCID_{50}$/10 L. However, the results are comparable to previous reports where 10^{-3} $TCID_{50}$/ml (equivalent to 10 $TCID_{50}$/10 L) were detected using a positively charged filter and ultrafiltration (Brassard et al., 2005); 0.2 pfu/ml of rotavirus (equivalent to 2×10^3 pfu/10L) were detected using an integrated cell culture and RT PCR system (Li et al., 2010) and 1.13×10^3 target copies/10L were detected through Nanoceram filtration and flocculation (Pang et al., 2012).

In contrast to the aforementioned methods, the resin-based method described here offers low cost (24 cents per 0.5g of resin), does not require specialized equipment, is not time consuming (the whole procedure of virus adsorption and nucleic acid isolation takes less than 2 hours) and it is simpler to perform. Direct isolation of nucleic acids from the resin allows for a very small volume of concentrated sample, making it possible to perform molecular detection on a substantial proportion of the original sample, as 10 L of water are reduced to 60 µl of final nucleic acid sample.

These advantages make this method promising as a simple and rapid method for viral surveys in tap and drinking water. Further studies with quantitative real time PCR are needed to assess the method capabilities; for example, adsorption efficiency and recovery rates would provide useful information for improvement of the method.

Chapter 7

Evaluation of a Simple and Cost Effective Filter Paper-Based Shipping and Storage Medium for Environmental Sampling of F-RNA Coliphages

7.1 Introduction

The family *Leviviridae* comprises a group of single stranded, positive sense RNA bacteriophages (F-RNA coliphages) that share structural characteristics with pathogenic human enteric viruses including caliciviruses, hepatitis A and E viruses, enteroviruses and astroviruses (Jofre et al., 2011). These F-RNA coliphages do not naturally proliferate in environments other than the gastrointestinal tracts of warm-blooded animals (Grabow, 2001), and their constant presence in human and animal feces make them suitable indicators of fecal contamination (Havelaar et al., 1993; Leclerc et al., 2000). The United States Environmental Protection Agency recognizes F-RNA coliphages as one of the three groups of microorganisms useful as fecal indicators (EPA, 2000), and they have been proposed as an index of the presence and risk of Norovirus in oysters and other bivalves (Doré et al., 2000), and as indices and indicators of viral contamination on animal carcasses (Flannery et al., 2009; Jones and Johns, 2012).

Based on their serological and genetic properties, F-RNA coliphages are classified into four genogroups within two genera: genogroups I and II are comprised within the genus *Levivirus*, and genogroups III and IV within the genus *Allolevivirus* (Leclerc et al., 2000). The presence or absence of particular F-RNA coliphage

genogroups can be used to track the origin of fecal pollution. F-RNA coliphage genogroups II and III are primarily associated with human sources of fecal contamination, while genogroups I and IV are predominantly associated with animal feces (Schaper et al., 2002b). However, some reports indicate that F-RNA coliphage/host associations are not absolute. Genogroups II and III were found in poultry, cattle, swine and dogs feces, and genogroups I and IV have been detected in human waste waters (Jofre et al., 2011; Noble et al., 2003; Scott et al., 2002).

A potential problem of this source tracking approach is the possible differential survival and recovery rates of different F-RNA phage genogroups in the laboratory. Highly sensitive and culture-independent molecular techniques based on RNA detection have been developed (Friedman et al., 2011; Kirs and Smith, 2007) to overcome these problems, but for such methods to be successful, shipment of the fecal samples from remote field settings and low resource regions to laboratory facilities must be adequate to maintain the suitability of specimens for molecular detection. Standard collection procedures of fecal material require fresh or frozen samples (Nechvatal et al., 2008), not only in order to avoid nucleic acid degradation but also to preserve the original profile of the sample microbiota (Cardona et al., 2012).

Filter paper is an appropriate alternative for collection, shipment and storage/preservation of virus-containing clinical samples. Several studies have described the use and efficacy of different types of filter paper (Maw et al., 2006; Michaud et al., 2007; Vilcek et al., 2001) to collect various clinical samples including blood, urine, and feces (Nozawa et al., 2007; Solmone et al., 2002) for detection of a wide variety of human and animal viruses including hepatitis A virus (Desbois et al.,

2009), hepatitis B (Lira et al., 2009), norovirus (Wollants et al., 2004) and rotavirus (Rahman et al., 2004). In general, viruses stored on filter paper have been shown to remain suitable for molecular detection for long periods of time. Nevertheless, molecular detection of some viruses is differentially impacted by storage time and/or temperature (Johansson et al., 1997; Katz et al., 2002; Rahman et al., 2004). To our knowledge, filter paper has not been evaluated as a medium for collection, storage/transport, and detection of low concentrations of F-RNA coliphages from feces.

The objective of this study was to compare the ability of filter paper as a storage and transport medium for F-RNA phages in feces to traditional cold storage transportation, and to determine the efficacy of filter paper as a simple and inexpensive method of sampling for the presence of F-RNA phages in remote areas and low resource settings. MS2 and Qβ were chosen for these analyses because they each represent one of the two genera (*Levivirus*, MS2; *Allolevivirus,* Qβ) of the family *Leviviridae* and are present in animal (genogroup I) or human feces (genogroup III).

7.2 Materials and methods

7.2.1 Coliphages and bacterial strains

F-RNA coliphages, MS2 (genogroup I) (ATCC 15597-B1) and Qβ (genogroup III) (ATCC 23631-B1), were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and amplified in *E. coli* HS (pFamp)R (ATCC 700891). Briefly, cultures of *E. coli* were grown in tryptic soy broth (supplemented with 50 µg/ml of ampicillin and streptomycin) to logarithmic phase and infected with coliphages at a multiplicity of

infection (MOI) of 5 to 10. Infected cultures were incubated with shaking for 18 to 24 hr, treated with chloroform (10%), and centrifuged at 6,000 × g for 25 min at 4°C to remove unlysed bacteria and cellular debris. The coliphage-containing supernatants were filtered through a 0.22 µm low protein-binding filter (PALL Life Sciences, Ann Arbor, MI) and stored at 4°C. Resulting coliphage stocks were enumerated using the double agar overlay plaque assay (Kropinski et al., 2008).

7.2.2 Inoculation of F-RNA coliphages in liquid samples and onto filter paper

Serial, ten-fold dilutions of coliphage stocks were performed in lambda buffer (liquid buffer samples) (0.58% NaCl, 0.2% $MgSO_4$ heptahydrate, 0.01% gelatin, and 0.05 M Tris-HCl, pH 7.5) or in 10% bovine manure slurry (liquid manure samples), made by homogenizing bovine manure (previously confirmed to be negative for the presence of F-RNA coliphages by real time RT-PCR) in lambda buffer. Liquid samples were stored at 4°C, and 10 µl aliquots were obtained at 0, 6, 13 or 37 days (three replicates were tested for each time point and treatment) for further RNA isolation.

Filter paper samples were prepared by spotting portions (10 µl) of each coliphage concentration from either the liquid buffer samples (filter paper buffer) or from the liquid manure samples (filter paper manure) onto 6 mm diameter, precut, Whatman No.1 filter paper circles (Whatman Inc., Clifton, N.J.). The filter paper samples were dried at room temperature for 1 hr, and packed into standard business paper 3 ⅝ × 5 ½ inches envelopes. Envelopes were sealed in a plastic bag and stored in an incubator at 37°C, to simulate transportation of the samples in a tropical region where refrigeration is

unavailable. As with the liquid samples, three replicates of each filter paper treatment were tested on 0, 6, 13, and 37 days post-incubation.

7.2.3 Environmental sampling

In addition to laboratory studies aimed at comparing storage methods, the use of filter paper as a storage medium for environmental fecal samples was also evaluated. Ten fresh bovine fecal specimens were obtained from a dairy farm in Northern China (Autonomous region of Xinjiang). Individual samples were placed in a plastic bag, diluted (1:10) in peptone water and 200 µl of the slurry was spotted on qualitative grade cellulose filter paper, equivalent to Whatman No 1 (New star, Hangzhou Fu Yang special paper Industry Co, Ltd, Hangzhou, Shanghai, China), dried overnight at room temperature, and stored in envelopes contained in plastic bags. After 10 days, RNA was isolated as described below, except that 140 µl of ultrapure water was added to a pool of three 6 mm filter paper disks punched out from the slurry spot. For negative controls, portions of clean filter paper areas surrounding the slurry spot were punched out and eluted as described (n=3 pools). Real time RT-PCR for all four coliphage genogroups was performed.

7.2.4 RNA isolation

RNA was obtained from equivalent amounts of coliphage liquid samples (10 µl) or from filter paper samples (one disk), to allow further direct comparison of results.

Nucleic acid isolation was performed using the QIAmp viral RNA kit© (Qiagen, Valencia, CA) according to the manufacturer's instructions. When liquid samples were used, 10 µl of each sample was mixed with 130 µl of water to attain the sample volume recommended by the kit manufacturer (140 µl). Alternatively, when filter paper samples were used, coliphages were eluted from filter paper prior to RNA purification by immersing each paper disk in 140 µl of nuclease free water and incubating it at room temperature for three minutes. The eluate was transferred to a clean 1.5 ml tube to start RNA purification. Final elution of RNA was accomplished using 60 µl of AVE buffer.

7.2.5 Nucleic acid detection

For the artificially inoculated samples, F-RNA coliphages were detected by real time RT-PCR using genogroup specific primers (Table 7.1) described by Kirs and Smith (Kirs and Smith, 2007). The reaction was modified to employ SYBR Green and OneStep real time RT-PCR kit enzymes (Qiagen, Valencia, CA) using the StepOne Plus thermocycler (Applied Biosystems, Foster City, CA). Real time RT-PCR reactions (15 µl) contained 5 µl of RNA, 0.6 µl of enzyme mix, 3 µl of 5× buffer, 100 nM of each forward and reverse primer, 0.4 mM (each) of deoxynucleotide triphosphates, 1.2 U RNAse inhibitor (Qiagen, Valencia, CA), and 0.3 µl of 10× SYBR Green solution (Sigma-Aldrich, Saint Louis, MO). Retrotranscription and thermocycling conditions were as follows: 30 min at 50°C, 15 min at 95°C, 40 cycles of 1 min at 95°C, 30 seconds at 60°C and 1 min at 72°C. Amplicon fidelity was evaluated through melting curve

analysis. Reaction products with the same melting temperature as purified coliphage controls (87.4°C for MS2 and 84.6°C for Qβ) were considered positive.

Real time RT-PCR detection of F-RNA coliphages from the naturally contaminated fecal samples was achieved using a probe-based assay (Table 7.1) specifically designed to detect a wide range of environmental F-RNA coliphages (Friedman et al., 2011). Reaction mixtures and cycling conditions were as described above with the following modifications: 600 nM of each forward and reverse primers for genogroup I, 800 nM for genogroup II, 400 nM for genogroup III and 800 nM for genogroup IV F-RNA coliphages, 300 nM of genogroup specific probes (5´ 6-FAM and 3´ Iowa Black FQ quencher) were used in each reaction (Table 1) (Integrated DNA Technologies, Inc., Coralville, IA).

7.2.6 Statistical analyses

Statistical analyses were performed using StatPlus LE.2009. Cycle threshold (Ct) means from liquid samples and filter paper samples were compared using a Student´s *t*-test. Linear regression was performed on filter paper Ct results over time. Pearson´s correlation was used to determine the association between real time RT-PCR results obtained from the liquid samples and those from the filter paper samples.

Table 7.1 Primer and probe sequences. Probes were labeled at the 5´end with 6-FAM and at the 3´end with Iowa Black FQ quencher. The standard IUB code was used for degenerated bases.

Genogroup	Name	5´- 3´ sequence	Source
I	K IFw	CGTGGTTCCATACTGGAGGT	Kirs and Smith 2007
I	K I Rev	CTTTCGAGCACACCCACC	
III	K III Fw	CCGCGTGGGGTAAATCC	
III	K III Rev	TTACGATTGCGAGAAGGCTG	
I	F I Fw	ATCCATTTTGGTAACGCCG	Friedman, Cooper *et al*. 2011
I	F I Rv	TGCAATCTCACTGGGACATAT	
I	F I probe	TAGGCATCTACGGGGACGA	
II	F II Fw	TTACTGTCGTTCCTGTTAGCAATG	
II	F II Rv	CRCCTGACGCACGATAACT	
II	F II probe	ACGGCGTCGCTGAGTGGCTTTC	
III	F III Fw	TAAATCCCACYAACGGYGTTGC	
III	F III Rv	TTICGATTRCGIGAAGGCTG	
III	F III Q like probe	TGGAGAAGCGTGTTACCGTTT	
III	F III M like probe	TGGAGAAGCGTGTYACAATTTCTGTRTC	
IV	F IV Fw	CGGYCAYCCGTCGTGGAAG	
IV	F IV Rv	AGTGACTGCTTTATTYGAAGTGCG	
IV	F IV probe	CCTGTCCGCAGGATGTWACCAAAC	

7.3 Results

7.3.1 Real time RT-PCR detection of coliphages RNA extracted from liquid samples and from spotted filter papers

To investigate how manure presence, and the process of spotting the sample onto filter paper affected the sensitivity of real time RT-PCR detection, RNA was obtained from liquid and filter paper samples prior to incubation (Time 0) and analyzed by real time RT-PCR. When RNA from liquid buffer samples was analyzed, the lowest concentration of coliphages detected was 10^1 pfu/sample for both MS2 and Qβ with average Cts of 35.5 and 33.2, respectively (Table 7.2). In contrast, in liquid samples containing manure, 10^1 pfu of coliphages were not consistently detected. For example, only one of three replicates was positive for MS2 (Ct = 36.3) and two of three replicates were positive for Qβ (average Ct = 34.7). Detection from filter paper samples was not consistent at the lowest (10^1 pfu/sample) F-RNA coliphage concentration, with only two of three replicates testing positive for coliphage MS2 in both the filter paper samples spotted with buffer or manure (average Ct = 34.9 and 35.2 respectively). All Qβ filter paper samples (buffer and manure) were not detected by real time RT-PCR (Ct > 40) at concentrations of 10^1 pfu/ml. However, in liquid and filter paper samples containing 10^2 to 10^4 pfu of coliphages, detection was always achieved for Qβ and MS2, even in the presence of manure. For example, detection of 10^2 pfu from filter paper buffer samples was achieved in about 33 cycles for MS2 and 34.2 cycles for Qβ, with higher concentrations of coliphages detected more rapidly (approximately 27 cycles in order to detect 10^4 pfu of either coliphage). Similarly, detection of 10^2 pfu of MS2 and Qβ from filter paper manure samples was achieved in approximately 35 cycles for MS2 and 34

cycles for Qβ, with higher concentrations (10^4 pfu/sample) detected at 31 cycles for MS2 and 25 cycles for Qβ.

Table 7.2 Average threshold cycle (Ct) and standard deviation (SD) of liquid and filter paper samples in different matrices at time 0. Each average represents three replicates. For the lowest coliphage concentration (10^1pfu/sample) superscripts indicate the amount of positive sample out the three replicates. When only one sample or no samples were positive, standard deviation was not calculated (N/A). Paired t-test (liquid vs. filter paper sample) was calculated for treatments with all three samples positives only (coliphage concentration $\geq 10^2$ pfu)

		Coliphage concentration/sample							
		10^1 pfu		10^2 pfu		10^3 pfu		10^4 pfu	
		Liquid sample	Filter paper	Liquid sample	Filter paper	Liquid sample	Filter paper	Liquid sample	Filter paper
MS2 in lambda buffer	Ct	35.5	34.9[a]	32.2	33	30.4	30.5	25.1	27.3*
	SD	0.21	0.63	0.7	0.7	1.2	0.2	0.2	0.2
MS2 in manure slurry	Ct	36.3[b]	35.2[a]	34.3	35.4	30.9	33.3*	27.7	31.2*
	SD	N/A	0.56	0.5	0.3	0.2	0.7	0.5	0.9
Qβ in lambda buffer	Ct	33.2	-[c]	30.4	34.2*	26.2	30.3*	22.8	26.6*
	SD	1.2	N/A	0.8	0.5	0.8	0.5	0.7	0.6
Qβ in manure slurry	Ct	34.7[a]	-[c]	28.9	33.6	26.8	30.1	24.2	25.4
	SD	2.79	N/A	0.7	0.8	0.5	0.6	0.7	0.4

[a] two out three samples were positive
[b] one out three samples was positive
[c] all samples were negative
* paired t-test liquid vs, filter paper sample p-value < 0.01

Comparisons between results of real time RT-PCR performed on liquid samples or alternatively on filter paper samples indicate slightly lower detection sensitivity in the filter paper samples. Differences in Ct ranged from 0.1 to 4.7 cycles, with few statistically significant differences (Table 7.2). However, at the lowest viral concentration where detection was achieved in all three replicates (10^2 pfu/sample), MS2 and Qβ detection was similar between filter paper and liquid samples for all treatments except for Qβ in buffer. Significant differences in real time RT-PCR detection for filter paper (p-value < 0.01) were observed for MS2 detection at high concentrations (10^3 and 10^4 pfu/sample) in samples containing manure and at 10^4 pfu/sample in samples without manure. Detection of Qβ in filter paper buffer samples was statistically different compared to detection in liquid buffer samples. In contrast, Qβ detection in filter paper manure samples showed no differences compared to liquid samples. On average, an increase in the real time RT-PCR threshold cycle due to spotting the sample on filter paper (ΔCt) (1.18 cycles for MS2 and 3.9 cycles for Qβ) was observed.

7.3.2 Effect of storage time and temperature on detection of F-RNA coliphages.

For MS2 filter paper samples, no statistical decrease in coliphage detection level was observed over time for any matrix (buffer or manure), at any coliphage concentration (p > 0.01), regardless of the incubation time (up to 37 days) at 37ºC (Figure 7.1). Qβ samples on filter paper were all readily detected by real-time RT PCR over 37 days at 37ºC, with no significant change in detection level over time, except for

filter paper samples inoculated with 10^4 coliphages in manure slurry, where a decrease in detection over time was observed ($p = 0.0056$). Strong correlations ($R \geq 0.869$) in detection were observed for coliphages stored in liquid matrices at 4ºC and coliphages preserved on filter paper at 37ºC, regardless of coliphage concentration and time of storage (Figure 7.2).

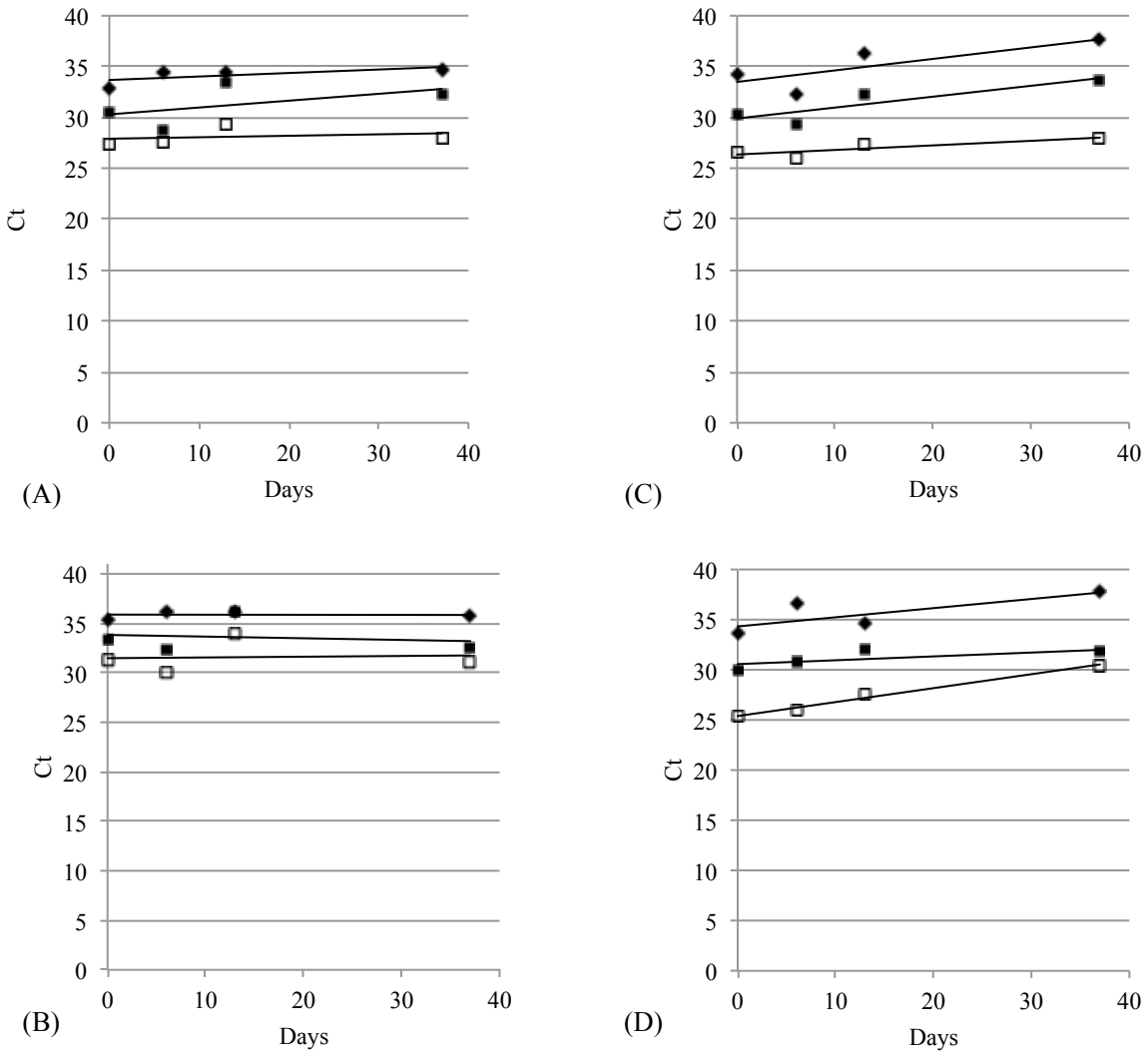

Fig 7.1 Mean Ct of filter paper samples stored at 37°C, inoculated with 10^2 pfu/sample (♦), 10^3 pfu/sample (▪) and 10^4 pfu/sample (▫). Samples were taken at 0, 6, 13, and 37 days. Filter paper of MS2 in buffer (A) or manure slurry (B). Filter paper of Qβ in buffer (C) or manure slurry (D). Linear regression p-values were all not significant (range from 0.113 to 0.958) except for Qβ 10^4 pfu/sample in manure slurry, where the p-value was 0.0

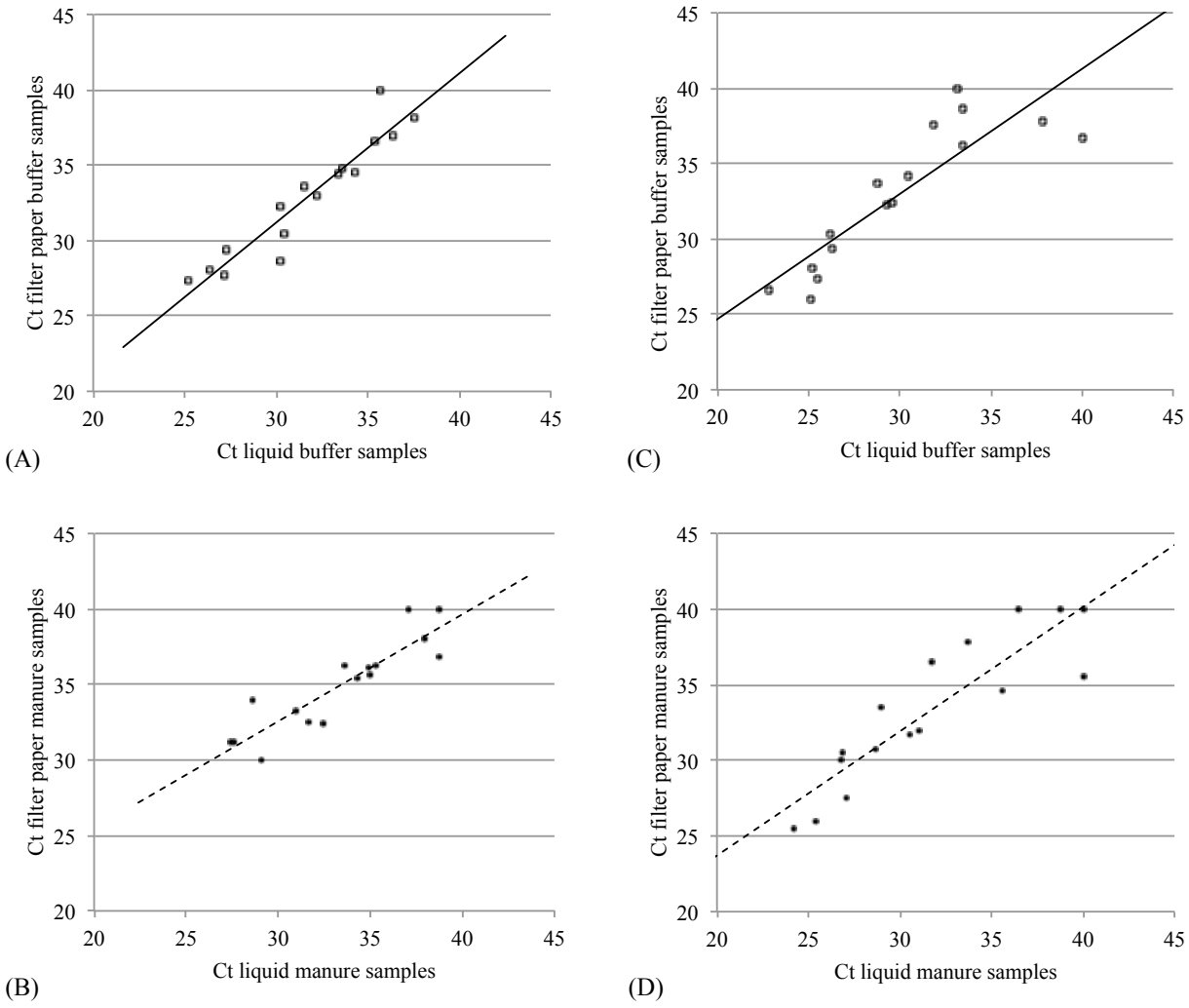

Fig 7.2 Correlation of real time RT-PCR results of samples maintained in liquid matrices (lambda buffer and manure slurry) at 4°C (x axis) with filter paper samples maintained at 37°C (y axis) for all virus concentrations and sampling times. (A) MS2 in buffer samples, (B) MS2 in manure samples, (C) Qβ in buffer samples, and (D) Qβ in manure samples. Pearsons correlation r = 0.947, 0.894, 0.869, and 0.890, respectively.

7.3.3 Environmental sampling

Real time RT-PCR for all four F-RNA coliphage genogroups was performed using RNA isolated from filter paper spotted with ten individual bovine fecal samples.

Since RT-PCR equipment was not available at the location where the bovine manure was collected, the filter paper samples were stored as described in section 2.3. The samples were kept at room temperature for 10 days following collection in Xinjiang, China, where day temperatures reached as high as 43ºC. As field samples may contain uncharacterized F-RNA coliphage strains, these samples were analyzed using another real time RT-PCR system proven to amplify a wide panel of environmental F-RNA coliphages (Friedman et al., 2011). Genogroup I (indicator of animal fecal contamination) F-RNA coliphages were found in 40% of the samples while no other genogroup (II, III or IV) coliphages were detected in any sample. Uninoculated filter paper controls tested negative for all four bacteriophage genogroups.

7.4 Discussion

Fecal source tracking using F-RNA coliphages has been accomplished by comparing the F-RNA coliphage genogroups present in feces of animal populations to the F-RNA coliphage genogroups present in impacted waters (Griffin et al., 2000; Lee et al., 2011). To achieve such objectives, it is necessary to directly sample fecal material and other environmental samples. As fecal material deteriorates easily at high temperatures due to high concentrations of metabolically active microorganisms, storage and transport of environmental fecal samples is currently required to be conducted at low temperatures (Cardona et al., 2012). However, the need to transport samples at refrigerated or lower temperatures to the laboratory for analysis can make it difficult to obtain samples from remote geographical areas, including developing

countries in which access to ice, and resources for timely shipment of the samples may be lacking. The results of this study support the use of filter paper as a simple and inexpensive alternative for storage and transport of fecal material samples at room temperature.

In this work, the real time RT-PCR detection sensitivity for coliphages MS2 and Qβ in liquid buffer samples (10^1 pfu) was similar to those previously reported by Kirs et al. (Kirs and Smith, 2007). However, the detection sensitivity for liquid manure samples and for samples spotted onto filter paper decreased (Table 7.2), where an additional 1 log of coliphages (10^2 pfu) were required for detection. The decrease in sensitivity of detection in manure samples and those on filter paper is likely due to the presence of PCR inhibitors in the fecal material, inefficient extraction of genetic material from the filter paper matrix, and/or viral damage caused by desiccation on the filter paper.

When results from the filter paper samples were compared to the corresponding liquid samples at time 0, some treatments showed lower sensitivity (Table 7.2). This is in agreement with reports on other RNA viruses (Abdelwhab et al., 2011; Desbois et al., 2009) where an increase of 2 to 4 cycles in the Ct was observed when RNA was isolated from the filter paper, compared to detection of RNA isolated from the original sample or dilutions of the virus stock. In our system, this loss of sensitivity was statistically significant for MS2 at high viral concentrations (10^3 and 10^4 pfu) and for Qβ only when the sample did not contain manure. For all treatments, the lowest coliphage concentration that was consistently detected in all three replicates after filter paper spotting was 10^2 pfu, a concentration that is within the range of coliphage concentrations found in fecal samples (Calci et al., 1998; Havelaar et al., 1986).

Although not always statistically significant, detection of Qβ from filter paper is more adversely effected than MS2 detection as evidenced by a larger increase in the ΔCt for Qβ samples (1.18 cycles for MS2 and 3.9 cycles for Qβ). This phenomenon may be explained by the differences in the susceptibility of the two phages to the desiccation process on the filter paper, or by differential binding of the two phages to the filter paper. In a study of comparative resistance of the four genogroups of F-RNA coliphages to various inactivation processes (Schaper et al., 2002a), the authors concluded that phages from genogroups III (Qβ) and IV were the least resistant to extreme temperatures, pH, salt concentration, ammonia, chlorine and natural inactivation in fresh water. Additionally, Schaper et al. demonstrated that genogroup I phages (MS2) showed the highest resistance to the aforementioned stresses. Nonetheless, the decrease in sensitivity did not have an effect on MS2 or Qβ detection at low (10^2 pfu/ml) concentrations in filter paper manure samples. As a result, and provided validation of the method with different strains of the four genogroups, the filter paper-based storage and transport approach can be reliably used for sampling feces for further downstream molecular detection of the F-RNA coliphages.

As a proof of concept, we performed a limited evaluation using filter paper to detect F-RNA coliphages by real time RT-PCR in bovine manure collected from a farm in Northern China. Previous studies have demonstrated the feasibility of storing viruses on many paper types (Maw et al., 2006; Michaud et al., 2007; Vilcek et al., 2001). Thus, an alternative to Whatman #1 filter paper was used for bovine manure sampling, as this was the only available option at the sampling location. Genogroup I F-RNA coliphages were detected in some of these bovine manure samples spotted on filter paper that

were kept at high, but variable, temperatures for 10 days, supporting the utility of the method. Since it was not feasible to isolate RNA and conduct real time RT-PCR on-site, it was impossible to assess the coliphage content in fresh samples. Such a comparison is needed to determine if filter paper storage differentially affected coliphage detection over time. Nevertheless, these results are in agreement with previous studies where 20% of bovine gastrointestinal contents (Osawa et al., 1981) and 6% of bovine waste waters (Cole et al., 2003) contained genogroup I F-RNA coliphages. Moreover, in these studies genogroup I F-RNA coliphages were the most abundant and omnipresent in positive samples.

Different inactivation rates of F-RNA coliphages genogroups have been reported when culture methods are used and the metric is infectivity. In survival experiments with the four F-RNA coliphage genogroups in seawater, Kirs et al. (2007) demonstrated that real time RT-PCR detection was possible after no infective phages were detected. Although F-RNA coliphage infectivity differentially waned between the four genogroups, molecular detection was not altered (Kirs and Smith, 2007), providing evidence that the genetic material may be a more stable target. In this work, two strains (corresponding to two F-RNA coliphage genogroups) were used to test the stability of molecular detection after filter paper inoculation. Based on these findings, small differences in the efficacy of molecular detection were noted between the two strains used. Therefore, future work with a broader panel of F-RNA coliphage strains is needed to fully validate the usefulness of the method.

In summary, upon inoculation onto filter paper, coliphages from feces remain detectable by real time RT-PCR for at least 37 days at 37ºC, providing opportunity to

transport samples from remote field sites to specialized laboratories for molecular detection and characterization, without the need for refrigerated storage and transportation. The strong correlation in real time RT-PCR detection between liquid manure samples stored at 4ºC and filter paper samples at 37ºC suggests that both storage systems are equivalent. Filter paper storage provides valuable advantages over refrigerated storage and transportation, including the fact that it is inexpensive (a few cents per sample), light weight, more compact and easy to handle compared to traditional storage and transportation techniques.

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Chapter 8

Concluding Remarks

Detection of viral pathogens or their viral surrogates in water samples is challenging because of a) a low concentration of the targets; b) a wide variety of water matrixes; c) the unpredictable sample content of inhibitors (in quantity and nature); d) and the need for cost effective and simple methodologies adaptable to continuous surveillance. The research project reported in this dissertation was designed to address some of the limitations of the currently used virus concentration/detection methods by proposing a novel alternative. The proposed methodology, although based on the same concept of adsorption of viruses by charge interactions as filter-based concentration methods, is different because it employs an anion-exchange resin. The nature of the resin beads selected for adsorption allows for water to flow around and through the beads in a batch-mixing format, instead of forcing the water sample through a filter, thereby avoiding technical problems such as clogging, breaking and leaking. Additionally, the spherical shape and porosity of the beads provide a very large adsorption surface in a small volume of resin, generating the possibility of using very small volumes of buffer for elution or lysis of the adsorbed viruses.

One possible disadvantage of a batch-mixing format is that stirring may be insufficient to generate enough opportunity for the virus particles to collide and adsorb to the resin beads. However, experiments to investigate the adsorption efficiency of the resin demonstrated that more than 95% of the F-RNA coliphages present in the water

sample were associated with the resin in less than 30 minutes (Chapter 4) providing support of the efficacy of the system to promote virus adsorption. Experiments measuring the residual virus titer of a viral solution after resin adsorption (therefore determining the resin adsorption efficiency alone) were performed using F-RNA coliphages only (Chapter 4), and the question of whether enteric viruses adsorb to the anion-exchange resin with similar high efficiency as F-RNA coliphages remains to be answered. Several facts support a speculative positive answer. These include the success of the resin-based method to increase sensitivity of molecular detection of adenovirus, hepatitis A virus and rotavirus (Chapter 6); the low detection limits (10 to 100 $TCID_{50}$/10L) of enteric viruses attained using this method (Chapter 6); and the structural similarities of F-RNA coliphages to enteric viruses and their wide use as enteric viruses models and indicators (Chapter 2).

When the entire resin-based concentration and RT-PCR detection procedure was tested with enteric viruses, the resin-based method yielded different results for different viruses (Chapter 6). As the experimental setting did not allow for determining which part of the concentration/detection method was the one impacted by virus differences, systematic studies targeting each aspect including adsorption efficiency, lysis efficiency and nucleic acid recovery separately are needed. The results from such studies will help to understand, manipulate and ultimately improve the resin-based method. Due to the fact that noroviruses are responsible for 99% of the viral gastroenteritis episodes caused by known agents (Scallan et al., 2011), experiments with this specific pathogen are warranted. As there is no *in vitro* propagation system for noroviruses, availability of samples with known viral content limited the possibility of

testing the resin-based concentration method with such pathogen. An alternative for future experiments would be the use of norovirus-like particles. These non-infectious particles are produced by self-assembling of recombinant norovirus capsid proteins, being antigenically and morphologically similar to the native norovirus capsids (Koho et al., 2012).

As showed in chapter 4, a more effective release of viral nucleic acids directly from the resin adsorbed viruses is required to obtain a better performance of the method. Systematic testing of different nucleic acid isolation procedures to determine the chemistry that provides the best release of nucleic acids of all different viruses tested will provide an important improvement to the method, retaining the advantage of no elution and small final sample volume.

The probable re-adsorption of nucleic acids to the resin once released from the capsids also warrants further investigation as its control may contribute to an increase in the efficiency of the resin-based methodology. Additionally, and probably more important, this information will allow us to speculate if this resin-based system addresses the frequently discussed problem of molecular techniques regarding detection of non-infectious particles. If naked nucleic acids are not adsorbed to the resin, the solely source of target for molecular detection would be viral particles. Resin-attached viruses holding nucleic acids inside would be most likely infective viruses, as loss of integrity of virus capsid contributes substantially to loss of infectivity (Knight et al., 2013). On the other hand, if the resin does adsorb nucleic acids (likely due to the DNA and RNA negative charge at neutral pH), treatment with RNAse and DNAse

enzymes prior to capsid lysis will provide the possibility to detect whole viruses only, increasing the chances for detection of mostly infectious particles.

Laboratory testing is the first step to develop any methodology, however experiments addressing real samples and field conditions for which the methodology is intended are essential, as demonstrated in Chapter 6. The first observation from environmental samples testing was the strong impact of the water matrix on the method performance. Despite the fact that the resin-based methodology was able to detect levels of F-RNA coliphage as low as 10^0 or 10^{-1} pfu/ml in tap water (Chapter 4), detection of 10^3 pfu/ml was completely inhibited for some field water samples and partially inhibited for the rest. Even though adsorption to the resin and molecular detection of F-RNA coliphages are both steps that could be affected by the water sample content, evidence presented here suggests that inhibition of molecular detection is the most affected process. Therefore, control of PCR inhibitors will contribute to noticeable improvement of the methodology. A possible approach for this control would be, taking advantage of the strong attachment of the viruses to the resin, to introduce washing steps for the resin after adsorption of the sample and prior to nucleic acid release. Additional strategies would be the treatment of the sample with activated carbon and/or use of phenol-chloroform nucleic acid extraction; choice of a more robust set of reverse transcription and DNA polymerase enzymes; or use of specific PCR additives such as betaine, bovine serum albumin, polyethylene glycol, powdered milk or T4 bacteriophage gene 32 product (gp32), proven effective against diverse PCR inhibitors (Schrader et al., 2012).

The variety of samples to be tested and thus the difficulty to design treatments in advance for unknown inhibitor content makes it likely that PCR inhibition would be still present in some instances. Therefore, an effective system to detect PCR inhibition is indispensable to assess the validity of the results with environmental samples. Another striking observation arising from the field-testing of the resin-based method was the ineffectiveness of the internal amplification control to predict the impact of the sample content on inhibition of molecular detection of the different targets tested. This observation supports the utility of using a spiked positive control for each sample tested (Chapter 6), as a practical alternative to detect false negative results. An additional advantage of this strategy is that it works as control for the whole concentration/detection method, not only for molecular detection. Although this strategy seems to be the most appropriate to mimic natural contamination, a potential failure is that spiked coliphages may be more available for resin adsorption than existing F-RNA coliphages as the later may have previously been adsorbed to solids present in the samples.

The results compiled in this dissertation provide enough evidence to support further research using the anion exchange resin-based virus concentration method. Basic research on the mechanisms of interaction of viral surfaces with the resin beads would provide information to predict and explain the limitations of the method. Additionally, applied research with more water samples, other viruses, other nucleic acid extraction methods and other PCR reactions would provide fast methodology improvements in order to validate its use for routine virus surveillance.

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