



**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**THE OCCURRENCE AND MOLECULAR
CHARACTERIZATION OF ENTERIC VIRUSES IN THE
TROPICAL AQUATIC ENVIRONMENT**

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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING
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THE OCCURRENCE AND MOLECULAR CHARACTERIZATION OF ENTERIC VIRUSES IN THE TROPICAL AQUATIC ENVIRONMENT

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ABSTRACT

Waterborne and water-related infectious diseases are a major cause of morbidity and mortality in countries at all levels of economic development. Most waterborne pathogens are introduced into drinking water supplies and recreational waters by human activities such as leaking sewage, urban runoff, agricultural runoff, and, in the case of estuarine and marine waters, wastewater discharge. Human enteric viruses can be of great concern because they are more environmentally stable as compared to bacteria, display resistance to various conventional methods used for controlling bacterial pathogens and cause infection at low infectious dose. Little is known about the epidemiology of enteric viruses as well as their occurrence in tropical aquatic environments, since most studies have been conducted in temperate and cold regions. The rapid improvements in molecular techniques now make it feasible to directly monitor and identify several important viral pathogens, particularly noroviruses, in water environments.

This thesis describes the first occurrence study on human enteric viruses in urban surface waters and wastewaters in Singapore. The prevalence and concentration of enteric viruses in the tropical aquatic environment were investigated by molecular techniques. The molecular characterization and phylogenetic analysis of environmental isolates were conducted to identify some of the dominant enteric viral strains in this geographical region.

Over a three-year study period, direct molecular detection of viruses in water samples after concentration by using tangential flow filtration revealed the prevalence of enteric viruses in urban catchments. Based on the positive detection rate of nested PCR assays, the distribution of enteric virus groups in urban surface waters was as follows: noroviruses GII > noroviruses GI > astroviruses > enteroviruses > adenoviruses > hepatitis A viruses. The molecular characterization of environmental viral isolates suggested co-circulation of multiple genotypes of both noroviruses GI and GII, astroviruses and enteroviruses in the aquatic

environment. Prevalent human viral genotypes detected in environmental water samples included norovirus GI/4, norovirus GII/4, astrovirus type 1, enteric adenovirus type 41 and coxsackieviruses A. Environmental isolates from surface waters were identical to or closely related to viral strains isolated from the community sewage, indicating that urban catchment areas are being impacted by human origin of fecal contamination. The binary logistic regression model suggested that bacterial indicators cannot be used to adequately predict the presence of enteric viruses in the tropical water catchments. The high prevalence of enteric viruses in water environments suggests the possible role played by water as a vector for viral transmission during recreational activities.

A comparative analysis was performed with environmental isolates of noroviruses and concurrently identified clinical isolates in Singapore during gastroenteritis outbreaks between August 2006 to January 2007. Genotyping of norovirus-positive clinical samples showed that all of the strains belonged to the GII/4 cluster. In contrast, genotyping of environmental samples showed a wide spectrum of genotypes of GI and GII, in addition to the GII/4 strains. The environmental and clinical norovirus GII/4 isolates showed high levels of nucleotide sequence identity to each other and to the novel GII/4 variant associated with global epidemics of gastroenteritis during 2006. The present results suggest that phylogenetic analysis of both environmental and clinical isolates is a useful approach in understanding the circulation of human pathogenic viruses within the community.

Outcomes of the present study allow more precise risk assessment for pathogenic waterborne viruses to be conducted in the tropical water environment and contamination prevention strategies to be addressed. The present findings call for a regular monitoring of urban water catchments for human enteric viruses and a routine clinical reporting on enteric viral infections. More information is needed on the epidemiology of potential waterborne viruses to fully understand the relationship between the occurrence of viral pathogens in the tropical environment and human health.

PUBLICATIONS

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LIST OF SYMBOLS / ABBREVIATIONS

AdV	Adenovirus
AstV	Astrovirus
ATCC	American Type Culture Collection
BGM	Buffalo green monkey
BLAST	Basic Local Alignment Search Tool
bp	base-pair
CaCo-2	Colonic carcinoma
CCL	Contaminant Candidate List
cDNA	Complementary deoxyribonucleic acid
CP	Crossing point
CPE	Cytopathic effect
CVA	Coxsackievirus A
CVB	Coxsackievirus B
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
FBS	Fetal bovine serum
HAV	Hepatitis A virus
HEK	Human embryo kidney
HEV	Human enterovirus
ICC	Integrated cell culture
IMS	Immunomagnetic separation
kb	kilo bases
kDa	kilo Dalton
LLCMK2	Continuous line of rhesus monkey kidney cells
MEM	Minimal essential medium
MgCl ₂	Magnesium chloride
MPN	Most probable number
mRNA	Messenger RNA

NV	Norovirus
NSP	Non-structural protein
ORF	Open reading frame
PBK	Primary baboon kidney
PCR	Polymerase chain reaction
PDU	Polymerase chain reaction - detectable units
PEG	Polyethylene glycol
PFU	Plaque forming unit
PV	Poliovirus
QPCR	Quantitative real-time PCR
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase - polymerase chain reaction
RV	Rotavirus
SAL	Single agar layer
ssDNA	Single-stranded deoxyribonucleic acid
ssRNA	Single-stranded ribonucleic acid
TCID	Tissue culture infective dose
TCVA	Total culturable viral assay
TFF	Tangential flow filtration
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
VP	Viral protein
WHO	World Health Organization
WRP	Water reclamation plant

Chapter 1

Introduction

1.1 An overview of emerging waterborne pathogens

A growing number of enteric microorganisms, including viruses, bacteria, protozoa and helminths, in polluted, untreated and treated waters have been identified as important waterborne pathogens (Straub and Chandler 2003; Ashbolt 2004). The presence of microbial pathogens in aquatic environments can cause significant deterioration of water quality and pose a public health threat. Microbial water quality often varies rapidly and short-term peaks in pathogen concentration may trigger outbreaks of waterborne disease. According to the World Health Organization (WHO), 1.8 million people die every year from diarrheal diseases, of which 88% of the disease is attributed to unsafe water supply, inadequate sanitation and hygiene (WHO 2004).

The potential of waters to transmit microbial pathogens is well documented in countries at all levels of economic development (Beller et al., 1997; Colwell et al., 2003; Lopman et al., 2003; Rangel et al., 2005; Karanis et al., 2007; Beaudreau et al., 2008). Between 1955 and 1956, the municipal water supply of New Delhi, India, was contaminated after the flooding of the river Yamuna, resulting in 35 000 cases of hepatitis E, with 73 deaths recorded among those infected (WHO 2001). The largest documented groundwater outbreak associated with coxsackievirus B3 and hepatitis A virus occurred in Texas, United States in June 1980 which affected approximately 7900 people (Hejkal et al., 1982). The massive outbreak of waterborne cryptosporidiosis in Milwaukee, Wisconsin, United States in 1993 which caused 54 deaths is another example of how microbial-contaminated water distributed through a municipal water system can result in significant public health and economic consequences in a community (Mac Kenzie et al., 1994). The continually increasing number of outbreaks that have been reported throughout the

world clearly demonstrates that transmission of pathogens via waters remains a major cause of illness.

In recent years, several so-called “emerging pathogens” have arisen as problems in watersheds supplying drinking water and identified as a public health risk (Szewzyk et al., 2000; Sharma et al., 2003). These include newly recognized pathogens from fecal sources such as enterohaemorrhagic *Escherichia coli* (*E. coli* O157:H7), *Campylobacter jejuni*, *Yersinia enterocolitica*, enteric viruses such as noroviruses, hepatitis E virus, adenoviruses and the parasites *Cryptosporidium parvum*, *Giardia Lamblia*, and Microsporidia. Emerging pathogens are those that have appeared in a human population for the first time, or have occurred previously but are increasing in incidence or expanding into areas where they have not been reported, usually over the last 20 years (WHO 2003). In 1998, the first Drinking Water Contaminant Candidate List (CCL) was produced by the United States Environmental Protection Agency (USEPA). The CCL contained a list of unregulated chemical and microbial contaminants which possess a potential risk for transmission through drinking water and thus, further research is needed to improve our understanding of their occurrence and fate in waters. The second CCL was published in 2005 (Table 1.1). Several of these pathogens on the current CCL have a very low infective dose and are resistant to environmental breakdown (Nwachuku and Gerba 2004).

Drinking and recreational water sources provide efficient modes for the spread of gastrointestinal pathogens capable of causing serious human diseases. There is continuous evidence of this causal connection worldwide. Emerging infectious diseases pose a particular problem in water supplies and will continue to challenge the water industry. Better knowledge of the occurrence, distribution, fate, and transport associated with the emerging potential waterborne pathogens in water environments would improve estimates of the overall pathogen risk for public health.

Table 1.1 Emerging waterborne pathogens on the United States Environmental Protection Agency Contaminant Candidate List 2 (CCL 2) associated with diseases caused

Emerging Pathogen	Major disease(s)
Bacteria	
<i>Aeromonas hydrophila</i>	Septicemia, diarrhea, wound infectious
Cyanobacteria (blue-green algae), other freshwater algae, and their toxins	Neuromuscular poison, hepatomegaly and liver necrosis
<i>Helicobacter pylori</i>	Gastric cancer
<i>Mycobacterium avium</i> intracellulare (MAC)	Tuberculosis
Protozoa	
Microsporidia (<i>Enterocytozoon</i> and <i>Septata</i>)	Diarrhea, cholangiopathy
Viruses	
Adenoviruses	Acute respiratory, gastroenteritis
Caliciviruses	Gastroenteritis
Coxsackieviruses	Meningitis, herpangia, myocarditis, paralysis
Echoviruses	Paralysis, diarrhea, meningitis

1.2 Enteric viruses in urban water environments: Current status

Enteric viruses have been recognized as potentially the most hazardous waterborne pathogens found in waters (Griffin et al., 2003). Waterborne enteric viruses are transmitted through faecal contamination in water from infected hosts and they can cause infection at low infectious dose, in comparison with other waterborne pathogens. There are more than 140 types of human enteric viruses (van Regenmortel et al., 2000). These include 72 serotypes of enteroviruses (poliovirus, coxsackievirus, echovirus), noroviruses (formerly known as the Norwalk-like viruses), adenoviruses, rotaviruses, hepatitis A viruses, hepatitis E viruses and astroviruses. Enteric viruses are associated with a variety of diseases such as gastroenteritis, hepatitis, myocarditis and meningitis in humans, especially the

immuno-compromised (Hedberg and Osterholm 1993; Cuthbert 2001). There may be many, as yet uncharacterized viruses that are responsible for gastrointestinal disease which may emerge in the future.

The role played by enteric viruses in the aquatic environment is less well understood compared to that of many bacteria and protozoa. This is partly due to the fact that no straightforward and reliable techniques are available for the detection of enteric viruses in waters. Moreover, enteric viruses constitute a special challenge in water and wastewater treatment processes due to their unique structure, composition and size. An increased understanding of the diversity and sources of pathogenic viruses and their persistence in water environments raises critical questions about the suitability of the current bacterial indicator approaches for assessing the human waste contamination of waters. Recent advances in molecular techniques make it timely to reassess the current paradigm of relying predominantly on traditional bacterial indicators for waterborne pathogens (Robertson and Nicholson 2005).

In highly urbanized water catchment areas, human pathogens, including enteric viruses, can be introduced from multiple pathways, which adversely affect receiving water quality. These pathways include urban stormwater runoff from nonpoint sources, discharge of untreated and treated wastewaters, combined sewer overflows and sanitary sewer overflows (Arnone and Walling 2007). Urban water catchments provide not only valuable resources for drinking water supplies, but also recreational water sources. Public health and recreational impacts such as the incidence of waterborne diseases related to recreational water contact and contamination of drinking water supplies are issues of concern.

Little is known about the prevalence, distribution and the fate of enteric viruses in aquatic environments in the tropical region, including Singapore, since most studies have been conducted in temperate and cold regions. As there is a strong focus on safe water supplies and water reclamation in Singapore, monitoring and identification of pathogenic viruses in recreational waters and sources of

drinking water are warranted. This in turn allows more confident and precise risk assessment associated with the use of different kinds of water to be conducted and contamination prevention strategies to be addressed.

1.3 Research questions, objectives and scope

This thesis describes the first study of the occurrence of human enteric viruses in urban surface waters and wastewaters in Singapore. It is aimed at providing important data to further our understanding of the true prevalence and molecular characterization of viral pathogens in the tropical aquatic environment. This study would have direct implications towards better surveillance of viral pathogens in waters, and thus public health protection. In this thesis, the following research questions are asked:

1. a) What are the patterns of distribution for human enteric viruses in urban surface waters and wastewaters in Singapore?
b) How do their occurrences correlate with concentrations of the conventional microbial indicators of water quality?
2. a) What are the genetic variations of the target isolated viruses in the tropical environmental water compared with those in the temperate and cold regions?
b) Could there be a spread of individual viral strains predominant in Singapore?
c) Could there be a relationship between the clinical and environmental viral isolates as determined by a genetic analysis?

Based on these research questions, the objectives of this study are as follows:

1. To use molecular techniques to study qualitatively and quantitatively, the occurrence and prevalence of human enteric viruses in untreated and treated urban wastewaters and surface waters from highly urbanized water catchment areas;
2. To characterize enteric viruses isolated from various environmental water and wastewater samples and to identify some of the dominant strains of waterborne viruses in this region by DNA sequencing and phylogenetic analysis;
3. To characterize noroviruses isolated from clinical samples during gastroenteritis outbreaks in Singapore and to determine the relationship between environmental norovirus strains with concurrently identified clinical isolates.

The scope of this thesis is limited to the examination of the virological quality of source waters for potential drinking water supply and recreational activities. Enteric viruses of concern in the present study are adenoviruses, astroviruses, enteroviruses, noroviruses and hepatitis A viruses.

1.4 Thesis outline

This thesis is organized into 6 different chapters. Chapter 2 is a comprehensive literature review of the detection and occurrence of enteric viruses in aquatic environments. Chapter 3 describes the materials and methods used in the present study. Chapter 4 provides results and detailed discussion on the occurrence and distribution of enteric viruses in tropical urban surface waters and wastewaters. Chapter 5 focuses on the molecular characterization and phylogenetic analysis of enteric viruses isolated from various environmental water samples. A comparative analysis between human noroviruses isolated from clinical samples during gastroenteritis outbreaks and environmental norovirus isolates is also presented. Chapter 6 summarizes important conclusions and outlines some recommendations for future study.

Chapter 2

Literature Review

2.1 Overview

This chapter is organized into six sections: (1) an overview of enteric viruses and their properties; (2) a description of waterborne outbreaks caused by enteric viruses; (3) and (4) are a review of current methodologies available for concentration, detection and quantification of enteric viruses in the aquatic environment; and (5) a discussion of the occurrence and distribution of enteric viruses in different water matrices.

2.2 Enteric viruses

Viruses are infectious agents that behave as obligatory intracellular parasites. They consist of a genetic material (RNA or DNA) enclosed in a protective protein coat or capsid. In the extracellular state, viruses are metabolically inert and do not carry out respiratory or biosynthetic functions. Nevertheless, viruses possess a degree of robustness which allows them to remain infectious outside of the susceptible host. This is well documented by the increasing number of outbreaks of waterborne disease caused by enteric viruses worldwide.

Enteric viruses (Figure 2.1) encompass all groups of viruses which may be present in the gastrointestinal tract; they may cause disease, or infection may be asymptomatic (Wyn-Jones and Sellwood 2001). The properties of human enteric viruses that may be transmitted by water are shown in Table 2.1.

Table 2.1 Classification and properties of enteric viruses of public health concern

Family	Genus	Principal species	Virion size (nm in diameter)	Genome	Disease caused
<i>Astroviridae</i>	<i>Mamastrovirus</i>	Human astrovirus (serotype 1 to 8)	28	Linear positive-sense single-stranded RNA (ssRNA), 6.8 – 7.2 kb in length	Gastroenteritis
<i>Adenoviridae</i>	<i>Mastadenovirus</i>	Human adenovirus (species A to F)	70 – 100	Linear double-stranded DNA (dsDNA), 36 kb in length	Gastroenteritis, respiratory disease, conjunctivitis
<i>Caliciviridae</i>	<i>Norovirus</i> <i>Sapovirus</i>	Norwalk virus Sapporo virus	27 – 30	Linear positive-sense ssRNA, 7.5 kb in length	Gastroenteritis
<i>Reoviridae</i>	<i>Rotavirus</i>	Human rotavirus	75	Linear double-stranded RNA (dsRNA), 16.5 kb – 21 kb in length	Gastroenteritis
<i>Picornaviridae</i>	<i>Hepatovirus</i>	Hepatitis A virus	30	Linear positive-sense ssRNA, 7.5 kb in length	Hepatitis
	<i>Enterovirus</i>	Poliovirus Coxsackievirus	27	Linear positive-sense ssRNA, 7.5 kb in length	Paralysis, meningitis, fever Hand-foot-and-mouth disease, meningitis, myocarditis
		Echovirus			Meningitis, gastroenteritis, fever, respiratory disease

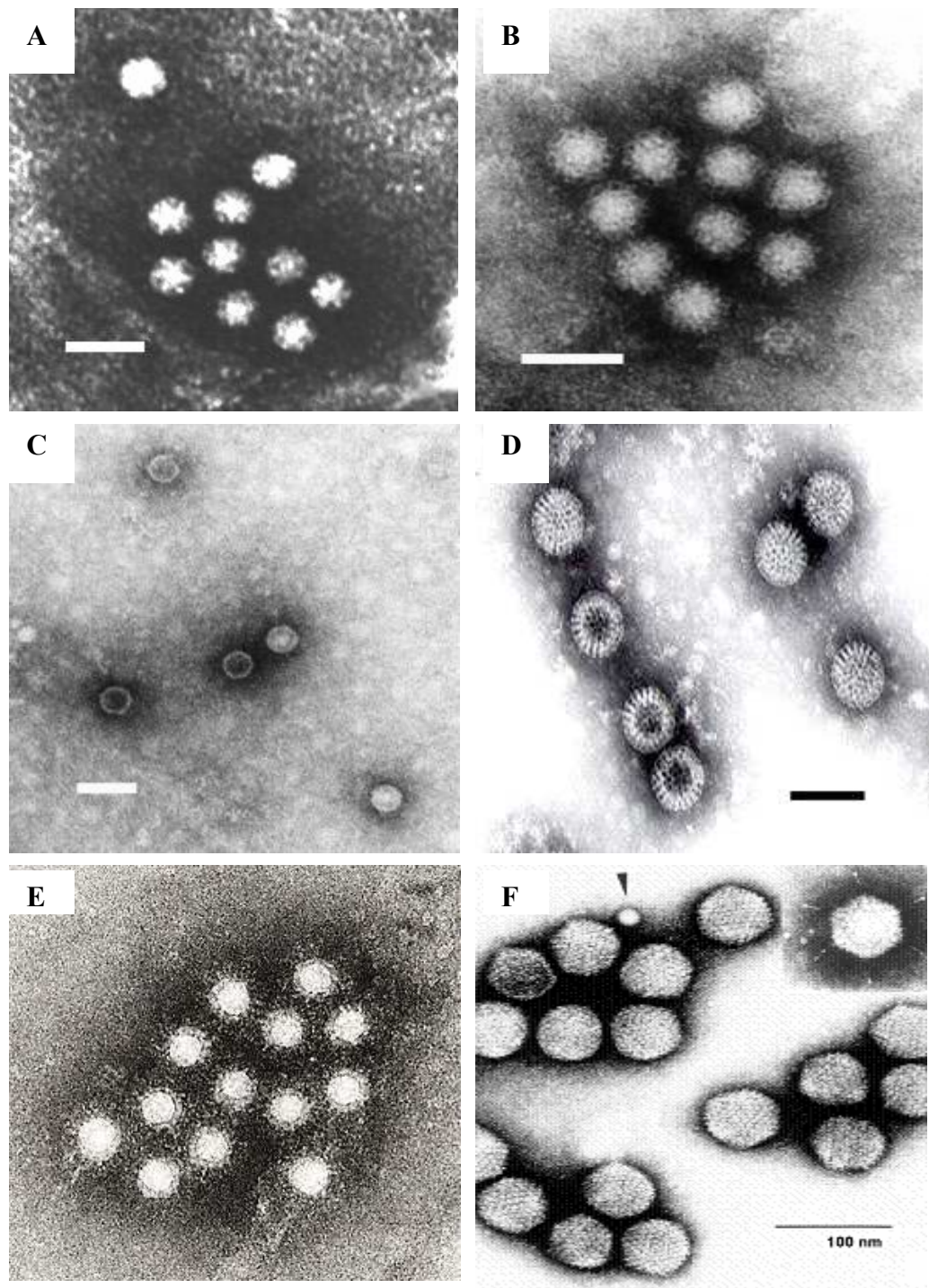


Figure 2.1 Transmission electron microscopy images of (A) astrovirus, (B) norovirus, (C) poliovirus, (D) rotavirus, (E) hepatitis A virus and (F) adenovirus particles. Images Sources: (A) to (D) F.P. Williams, U.S. EPA; (E) Centers for Disease Control and Prevention, U.S.; (F) International Committee on Taxonomy of Viruses.

2.2.1 Enteroviruses

Enteroviruses are non-enveloped icosahedral particles with no obvious surface structure, as seen by electron microscopy. There are four species of human enteroviruses (HEV-A to HEV-D) in the genus *Enterovirus* of the *Picornaviridae* (Table 2.2). Each species contains several serotypes: polioviruses, coxsackieviruses A, coxsackieviruses B, echoviruses and the newer enteroviruses given names with a system of consecutive numbers (see Table 2.2) (Hovi et al., 2007). The genome of enteroviruses contains a single open reading frame (ORF) from which the various proteins are derived. Environmental virology began with efforts to detect poliovirus by cell culture methods in sewage and water more than 60 years ago (Metcalf et al., 1995). Enteroviruses are the viruses most frequently detected in polluted water and can be transmitted by the fecal-oral route.

Table 2.2 Classification of human enteroviruses (HEV)

Species	Serotypes ^{a, b}
HEV-A	CVA2-8, 10, 12, 14, 16; EV71, EV76, 89-91
HEV-B	CVA9; CVB1-6; E1-7, 9, 11-21, 24-27, 29-33; EV73-75, 77-79, 81-83, 85-86, 97, 100
HEV-C	PV1-3; CVA1, 11, 13, 17, 19-22, 24; EV96
HEV-D	EV68, 70, EV94

^a CVA, coxsackievirus A; CVB, coxsackievirus B; EV, enterovirus; E, echovirus; PV, poliovirus.

^b Newer enterovirus types identified by molecular methods are in bold font.

2.2.2. Rotavirus

Rotaviruses are relatively large icosahedral virions with a triple layer of protein. The complete particle has a wheel-like appearance. Rotaviruses possess a genome of 11 segments of double-stranded RNA. The genome of rotavirus codes for six structural viral proteins (VP1, VP2, VP3, VP4, VP6 and VP7) and six non-

structural proteins (NSP1-6). There are six serogroups, termed groups A-F recognized based on the inner capsid antigen, VP6. Rotaviruses have also been characterized on the basis of genogroups (Baggi and Peduzzi 2000). Rotaviruses have been recognized as the major cause of infantile gastroenteritis worldwide. During August 2001 to July 2002, 45% of acute diarrheal hospitalizations among children 0-5 years in Asia were attributable to rotavirus, higher than previous estimates (Bresee et al., 2004).

2.2.3 Astroviruses

Astroviruses are small, non-enveloped viruses with a characteristic five- or six-pointed star-shaped motif on the particle surface. The genome of astroviruses is arranged into three open reading frames (ORF1a, 1b and 2). Astroviruses were first discovered by electron microscopical examination of stool specimens from babies with gastroenteritis by Appleton and Higgins (1975). There are currently eight recognized serotypes of human astroviruses with serotype 1 being the most globally prevalent. Good correlation between antigenic and genomic types of human astrovirus has been shown by nucleotide sequence analysis of a partial region of the ORF2 (Noel et al., 1995). Among the viruses recognized as agents of viral gastroenteritis, the role of astroviruses in the aquatic environment is perhaps the least well understood (Percival et al., 2004a) .

2.2.4 Adenoviruses

Human adenoviruses are members of the *Mastadenovirus* genus, which have very distinct icosahedral structures when visualized by electron microscopy. The capsid comprises 252 capsomeres of which 240 are hexons and 12 are pentons. Each penton base of the capsid has a glycoprotein fiber that aids in the attachment to the host cell. Adenoviruses are the only enteric viruses composed of double-stranded DNA which is about 36 kilobases and encodes more than 30 structural and non-structural proteins. There are currently 51 recognized serotypes of human adenoviruses, which can be classified into six species, designated species A to F.

Species F contains two fastidious serotypes, 40 and 41, which have been recognized as the second most important etiological agents of viral gastroenteritis in children (Horwitz 2001). Assessing the health risk posed by adenoviruses in water is hampered by a lack of information on the infectious dose of enterically transmitted adenoviruses (Yates et al., 2006).

2.2.5 Caliciviruses

The *norovirus* and *sapovirus* genera of the *Caliciviridae* family contain human and animal virus strains. Calicivirus particles have a ragged outline and the capsid surface is a series of arches and cup-like depressions. The capsid is made of a single protein arranged in icosahedral symmetry. The genome of caliciviruses is arranged into three major ORFs that encode non-structural, capsid and minor structural proteins, respectively (Lambden et al., 1993). Noroviruses which have been previously termed Norwalk viruses or small round structured viruses are a major cause of acute non-bacterial gastroenteritis across all age groups worldwide (Patel et al., 2009). Phylogenetic characterization of sequence information of the genes encoding the viral RNA-dependent RNA polymerase (RdRp) and the capsid protein divides the norovirus genus into five genogroups (GI to GV) (Zheng et al., 2006). Each genogroup can be further divided into different genotypes, of which GI (8 genotypes), GII (17 genotypes) and GIV (1 genotype) genotypes are mostly human pathogens.

Noroviruses are highly infectious and transmitted primarily via the fecal-oral route. Foodborne outbreaks of acute gastroenteritis associated with noroviruses have been frequently reported worldwide, including Singapore (Lopmam et al., 2003; Kageyama et al., 2004; Ng et al., 2005; Widdowson et al., 2005; Webby et al., 2007). The inability to propagate noroviruses in conventional cell culture or in animal models has hampered our understanding of virus replication and their stability to environmental inactivation and water treatment processes.

2.2.6 Enteric hepatitis virus

Hepatitis transmitted via the fecal-oral route represents the most common manifestation of acute hepatitis worldwide (Pinto and Saiz 2007). The enteric hepatitis (formerly known as infectious hepatitis) includes two types: hepatitis A and hepatitis E.

The etiological agent of hepatitis A is the hepatitis A virus which belongs to the genus *Hepatovirus* within the *Picornaviridae* family. The non-enveloped virion of hepatitis A virus has no obvious morphology by electron microscopy. The genome of hepatitis A virus contains a single ORF which may be divided into three functional regions, termed P1, P2 and P3. The P1 genomic region encodes the capsid polypeptides and the P2 and P3 regions encode the non-structural polypeptide necessary for viral replication. Hepatitis A virus is difficult and slow to grow in cell culture, thus the detection of hepatitis A virus in water has relied mainly on molecular means (Percival et al., 2004b). The causative agent of hepatitis E, formerly known as enterically transmitted non-A, non-B hepatitis, was first described in 1990 (Reyes et al., 1990). Hepatitis E virus is a naked viral particle with little surface structure visible by electron microscopy. Most epidemics of hepatitis E virus are associated waterborne outbreaks, particularly in developing countries with poor sanitary conditions and inadequate water supplies (Wong et al., 1980; Pinto and Saiz 2007).

2.3 Waterborne disease outbreaks associated with enteric viruses

Waterborne disease outbreaks are normally associated with the consumption of contaminated drinking water or participation in water-related activities in contaminated recreational waters. The challenge of proving that waterborne outbreaks of disease are attributable to enteric viruses is much greater than that associated with bacterial or protozoan disease. One of the major obstacles is the difficulty in detecting and enumerating enteric viruses directly in water samples.

However, recent advances in molecular techniques significantly improve the ability to detect the causative agents in environmental samples and thus, improve our knowledge regarding viral etiology in waterborne disease outbreaks. Several outbreaks of waterborne viral disease that have been documented with etiologic agents could be traced both in patient stool and water samples by using molecular techniques (Table 2.3).

The earliest recreational waterborne outbreak caused by coxsackievirus B5 was documented in Vermont, United States, with the virus isolated for the first time from the lake water during the outbreak (Hawley et al., 1973). A more recent water-related enterovirus outbreak was reported in the United States in June 2004 (Begier et al., 2008). The authors identified cases of concurrent echovirus 30 and coxsackievirus A1 infections associated with sea swimming among a group of travelers to Mexico. The report highlights for the first time, a novel mode of transmission of viral meningitis through recreational activities in contaminated seawater. Unfortunately, the contaminated seawater was not sampled and tested for enteroviruses due to the distance and time constraints (Begier et al., 2008). Amvrosieva et al. (2006) reported the drinking water associated meningitis outbreak in the Republic of Belarus in 2003 with echovirus 30 as the major causative agent. The authors revealed high levels of genetic similarity between the clinical and environmental isolates during the outbreak, suggesting the possibility of transmission of infections caused by enteroviruses via drinking water.

Outbreaks of adenovirus infection have been reported mainly in swimming pools, with non-enteric adenoviruses as major causative agents (Dangelo et al., 1979; Papapetropoulou and Vantarakis 1998). Failure of chlorination is often the reason that triggers the start of waterborne adenovirus outbreaks in swimming pools. Enteric adenoviruses were identified as one of the agents causing acute gastroenteritis in a waterborne outbreak associated with contaminated drinking water in Finland (Kukkula et al., 1997).

Table 2.3 Examples of recent waterborne viral outbreaks in which etiologic agents have been identified both in patients and water samples

Outbreak period	Location	Source and cause of outbreak	Etiologic agent(s) ^a		Reference
			Patients	Water	
Summer 1995	Quebec, Canada	Drinking water source from well, fecal contamination due to multiple fractures in the bedrock	HAV genotype IA	HAV genotype IA	De Serres et al. (1999)
July 1995	Southern Greece	Swimming pool, chlorination failure	AdV	AdV	Papapetropoulou and Vantarakis (1998)
August-September 1998	La Neuveville, Switzerland	Drinking water source from groundwater, sewage leakage	NV GI, GII	NV GI	Maurer and Sturchler (2000)
1998-2003 (18 norovirus outbreaks)	Finland	Drinking water source from surface water (3 cases) and groundwater (15 cases), inadequate disinfection, sewage leakage	NV GI/3, 6, GII/4, 5, b, d	NV GI/3, 6, GII/1, 4	Maunula et al. (2005)
August 2000	Gourdon, France	Drinking water source from groundwater, chlorination failure	RV A genotype G1, NV GI, GIIB	RV A genotype G1	Gallay et al. (2006)
February 2001	Wyoming, United States	Drinking water source from well, overloaded sewage disposal system	NV GII	NV GII	Anderson et al. (2003)

Table 2.3 (Continued)

Outbreak period	Location	Source and cause of outbreak	Etiologic agent(s) ^a		Reference
			Patients	Water	
July 2001	Helsinki, Finland	Contamination of wading pool water with fecal material	NV GII/4, AstV	NV GII/4, AstV	Maunula et al. (2004)
October 2001	Wyoming, United States	Well water, chlorination failure	NV GI/3, GII/6	NV GI/3	Parshionikar et al. (2003)
July-October 2001	Kassel, Germany	Nature-like swimming pool	E13, 30	E30	Hauri et al. (2005)
May-June 2001	Stockholm, Sweden	Well water, sewage overflow	NV GIIb	NV GIIb	Nygard et al. (2003)
June 2002	The Netherlands	Recreational water fountain	NV GI/3	NV GI/3	Hoebe et al. (2004)
June-September 2003	Central Italy	Leakage of fecal contamination groundwater and seawater into drinking water system	NV GI/3, GII/4	NV GII/4	Migliorati et al. (2008)
July-November 2003	Minsk, Republic of Belarus	Drinking water	E30, E6, CV B5	E30, E6, CV B5	Amvrosieva et al. (2006)
May 2004	Jeju Island, South Korea	Water supply from groundwater, external contamination due to cracks in the borehole	NV GI/3, 4, 6, GII/4, 5, 8, 14	NV GI/1, 3, GII/3, 6	Kim et al. (2005)

Table 2.3 (Continued)

Outbreak period	Location	Source and cause of outbreak	Etiologic agent(s) ^a		Reference
			Patients	Water	
May 2006	Sichuan Province, China	Ice snacks made from sewage contaminated well water	HAV genotype IA	HAV genotype IA	Zhang et al. (2009)
July 2006	New Zealand	Drinking water source from natural spring, sewage overflow	NV GI/5	NV GI/5	Hewitt et al. (2007)
August 2006	North Carolina, United States	Drinking water source from shallow spring, contamination from septic tank	HAV genotype IA	HAV genotype IA	Tallon et al. (2008)

^a AdV, adenovirus; AstV, astrovirus; CV, coxsackievirus; E, echovirus; HAV, hepatitis A virus; NV, norovirus; RV, rotavirus.

A viral etiologic agent, known as Norwalk virus, for outbreaks of acute infectious non-bacterial gastroenteritis was confirmed by electron microscopy studies in 1972 (Kapikian et al., 1972). This viral pathogen has been subsequently shown to be a frequent cause of outbreaks of foodborne and waterborne acute infectious non-bacterial gastroenteritis worldwide (Hedberg and Osterholm 1993; Lopmam et al., 2003). In recent years, an increasing number of reports have described waterborne gastroenteritis outbreaks caused by noroviruses, with norovirus genotypes that could be well identified by phylogenetic analysis of partial genomic sequences (Table 2.3). Sewage-contaminated surface water and groundwater sources have been implicated in numerous drinking water-associated norovirus outbreaks, suggesting the ability of noroviruses to remain infective in natural water environments. More recently, Werber et al. (2009) reported a massive viral gastroenteritis outbreak associated with a consumption of drinking water from chlorinated water supplies. Noroviruses were identified as main causative agents of the outbreak. The report demonstrates that sustained vigilance of municipal water distribution systems is essential including those with established chlorination supply. Contaminated recreational waters as modes of transmission of norovirus infections have also been demonstrated in several outbreaks worldwide (Baron et al., 1982; Kappus et al., 1982; Gray et al., 1997; Maunula et al., 2004; Podewils et al., 2007; Sartorius et al., 2007).

Most hepatitis A outbreaks are associated with the consumption of raw shellfish grown and harvested from waters receiving urban contaminants (Halliday et al., 1991; Sanchez et al., 2002). Nevertheless, several waterborne outbreaks of infectious hepatitis caused by contaminated drinking water and recreational waters have also been documented (Bryan et al., 1974; Bloch et al., 1990; Mahoney et al., 1992; De Serres et al., 1999; Zhang et al., 2009). A recent investigation of an acute waterborne hepatitis A outbreak in North Carolina, by using phylogenetic analysis of hepatitis A virus genomic sequences to determine the source of exposure, has proven the usefulness of molecular techniques by providing timely information to assist with public health prevention and control measures (Tallon et al., 2008).

2.4 Methods for the concentration of enteric viruses

Analysis of water for enteric viruses usually consists of concentration/enrichment, detection, identification, and quantification. Concentration is necessary because the ambient concentrations of viruses in environmental waters are usually less than the limit of detection. The volume of water sample to be processed depends upon the level of viruses likely to be present in the water. For example, when virus adsorption-elution method is used, recommended sample volumes for sewage, surface water and groundwater or finished drinking water are 2 to 20 liters, 200 to 300 liters and 1500 to 2000 liters, respectively (USEPA 2001). An ideal concentration method should fulfil the following criteria to be of practical use: be technically simple, rapid and inexpensive, provides high virus recovery rate in a small volume of final concentrate and be able to concentrate a wide range of virus groups simultaneously. However, no single concentration method meets all these requirements (Wyn-Jones and Sellwood 2001).

The first stage of concentration is normally comprised of a technique such as adsorption-elution or ultrafiltration which reduces the initial volume of water sample to between 100 and 500 ml. The secondary concentration is needed for reconcentrating viruses to a sample volume of less than 10 ml. Different techniques for virus concentration from water samples are summarised in Table 2.4 and are reviewed in the following sub-section.

2.4.1 Adsorption-elution

For adsorption-elution techniques, viruses will adsorb under specific conditions of pH and ionic strength to charged matrices. Viruses are then eluted from the matrix into a smaller volume using glycine buffer or a solution containing beef extract or skimmed milk. Secondary concentration is then used to further concentrate the eluant containing viruses. Examples of adsorbing matrix are glass wool, glass powder, positively and negatively charged membranes.

Table 2.4 Concentration methods for enteric viruses in water samples

Method (Principle)	Virus recovery (Reference)	Advantages	Disadvantages
Adsorption-elution (Ionic charge) Glass wool	- 62-75% for EV, tap water (Vilagines et al., 1992); - 14-70% for EV, 21% for AdV, 29% for NV, tap water (Lambertini et al., 2008)	- Inexpensive; - Suitable for viruses sensitive to acid; - No pre-conditioning of sample is required	- Elution with beef extract, potential inhibitory to RT-PCR
Glass powder	- 48% for EV, treated wastewater (Gantzer et al., 1997); - 61-100% for HAV, sewage, tap water and surface water (Gajardo et al., 1991)	- No secondary concentration is required for low sample volume	- Complexity of the apparatus, impractical
Positively charged membrane	- 27-54% for EV, tap water (Karim et al., 2009)	- Suitable for viruses sensitive to low pH conditions; - No pre-conditioning of sample is required	- Organic matters, especially fulvic acid may interfere virus recovery; - Costly
Negatively charged membrane	- 62% for poliovirus, seawater (Katayama et al., 2002); - 9-43% NV, AstV, river water (Victoria et al., 2009)	- Can be used with alkaline elution step instead of beef extract	- Pre-conditioning of sample is required; - Clogging for turbid water
Immunomagnetic separation (Antibody capture)	Data not available	- Remove RT-PCR inhibitors from concentrated environmental water; - Increase specificity for the downstream detection assay	- Few data available, undeveloped; - Specific assay is required for each virus

Table 2.4 Continued

Method	Virus recovery (Reference)	Advantages	Disadvantages
Ultrafiltration (Particle size separation) Tangential flow filtration	- 49% for echovirus, tap water (Hill et al., 2005); - 52-82% for poliovirus, river water (Olszewski et al., 2005)	- No pre-conditioning of sample is required; - Suitable for recovering a wide range of viruses simultaneously	- Final retentate volume depends on the apparatus in use; - Costly; - Clogging for turbid water – longer processing time
Precipitation (chemical precipitation) Organic flocculation	- 75% for poliovirus, tap water concentrates from adsorption-elution (Katzenelson et al., 1976)	- Well established secondary concentration step	- Low pH condition required may inactivate viruses - Beef extract may co-precipitate with viral nucleic acid and inhibit the RT-PCR assay
Polyethylene glycol	- 71-88% for EV, 75% for RV, tap water concentrates from adsorption-elution (Vilagines et al., 1997b)	- No pH manipulation of the sample, suitable for viruses sensitive to low pH; - Able to obtain precipitation at neutral pH and high ionic concentrations	- Impractical for large volumes of water samples
Ultracentrifugation (Physical sedimentation)	Data not available	- Suitable as secondary concentration; - Wide range of viruses can be recovered simultaneously; - More rapid for concentrating viruses in sewage compared to chemical precipitation	- Limited volumes can be processed; - Costly, lack of portability of equipment

Glass wool offers an economic alternative as an adsorptive material for virus concentration. It is evenly packed in a column and is able to attract negatively charged virus particles at or near neutral pH. A round robin test of the glass wool method conducted in France, for the concentration of polioviruses from drinking water and seawater, showed the average recovery efficiencies from 72 to 75% (Vilagines et al., 1997a). Recently, this method has been demonstrated by Lambertini et al. (2008) to be effective in recovering four enteric viruses on the USEPA CCL from drinking water, although the recovery efficiencies were highly variable.

Glass powder constitutes a fluidized bed and can thus prevent clogging of the filter matrix during concentration processes. Sarrette et al. (1977) developed this technique to concentrate enteroviruses from tap water, which was later extended for hepatitis A viruses in different environmental matrices (Gajardo et al., 1991). Complexity of the apparatus renders this technique impracticable for routine field surveys of viruses in environmental waters.

Concentration of viruses in environmental waters by the adsorption-elution with positively and negatively charged membranes has been practised for many years (Enriquez and Gerba 1995; APHA 1998). Positively charged membranes adsorb viruses from water in the pH range 3 to 6. This makes possible the concentration of viruses such as rotavirus and coliphages, which are sensitive to the low pH conditions needed for adsorption to negatively charged membranes (Borrego et al., 1991; Gilgen et al., 1997; Brassard et al., 2005). However, positively charged membranes are easily clogged and have low recovery efficiency for viruses in seawater due to the presence of salt and alkalinity of seawater (Lukasik et al., 2000). Electropositive filters 1MDS (Cuno) are the most commonly used filters for concentrating enteric viruses from water. However the 1MDS filters are not cost effective for routine virological monitoring of water. A recent comparative study between these filters and inexpensive NanoCeram (Argonide) filters suggests that NanoCeram filters can be used as an alternative to 1MDS filters for routine viral analysis (Karim et al., 2009).

Negatively charged membranes show higher virus recoveries from seawater and waters of high turbidity compared with positively charged membranes (Lukasik et al., 2000). A new procedure for concentrating enteric viruses from seawater using a negatively charged membrane has been developed by Katayama et al. (2002). This protocol is free from beef extract elution, which has an inhibitory effect in the RT-PCR detection of viral genomes and was demonstrated to have recovery rates greater than 61% for poliovirus in seeded seawater. The protocol was further optimized by Haramoto et al. (2004) and found to be useful for measuring the occurrence of enteric viruses, including noroviruses, in large volumes of freshwater. There is an increasing trend to use negatively charged membranes for concentrating enteric viruses in small volumes of environmental waters (2 liters or less) for molecular detection (Fong et al., 2005; Fuhrman et al., 2005; Gersberg et al., 2006; Chen et al., 2008b; Hamza et al., 2009). A recent finding by Victoria et al. (2009) suggests that a specific concentration of $MgCl_2$ is needed for each type of environmental water in order to obtain the best recovery of enteric viruses by negatively charged membranes. The addition of $MgCl_2$ to environmental water is believed to permit virus adsorption and retention onto negatively charged membranes.

2.4.2 Ultrafiltration

Ultrafiltration uses size-exclusion as the mechanism of concentration, where molecules that are smaller than the filter pore size pass through the filter while larger compounds are withheld in the retentate. Ultrafiltration such as tangential flow filtration (TFF) with cassette membrane system (Soule et al., 2000; Choi and Jiang 2005; Petrinca et al., 2009) and hollow-fibre system (Winona et al., 2001; Morales-Morales et al., 2003; Olszewski et al., 2005) have been used to concentrate enteric viruses and coliphages in various water matrices .

One of the advantages of ultrafiltration techniques is that a wide range of viruses can be recovered in a single water sample, including viruses sensitive to the pH changes necessary for most adsorption-elution techniques. Increased regulatory

focus on microbiological quality of waters has driven the development of rapid and streamlined techniques for the concentration and detection of multiple human pathogens in water. Several studies have demonstrated that ultrafiltration can be effective for simultaneously recovering various microorganisms, including *Cryptosporidium parvum* oocysts and viruses, from water samples (Morales-Morales et al., 2003; Hill et al., 2005; Polaczyk et al., 2008; Hill et al., 2009; Holowecky et al., 2009).

2.4.3 Immunomagnetic separation

In the past few years, immunomagnetic separation (IMS) has been introduced as an alternative for purification of pathogens in environmental water samples before subsequent detection assay (Safarik and Safarikova 1999). This technique, using combinations of various magnetic beads and antibodies against specific viral surface antigens, has been used to isolate hepatitis A virus, rotaviruses, enteroviruses (Jothikumar et al., 1998; Casas and Sunen 2002; El Galil et al., 2004; Hwang et al., 2007), and noroviruses (Myrmel et al., 2000; Cannon and Vinje 2008) in seeded environmental water samples. It has been suggested that this antibody capture provides an approach for the detection of intact and thus potentially infectious viruses because antibodies only capture those viral particles with functional antigenic epitopes on the viral surface (Schwab et al., 1996). However, IMS is probably not economical and practical for large volumes of water sample and their application to virus concentration from field samples has not been extensively demonstrated.

2.4.4 Secondary concentration methods

Organic flocculation is the most common secondary concentration method when a protein solution such as beef extract or skimmed milk is used as the eluant. This method was first described by Katzenelson et al. (1976), which is performed by lowering the pH of the eluant to 3.5 in order to produce protein precipitates. The virus-containing floc can subsequently be collected by centrifugation and dissolved

in a small volume of buffer. Although good recoveries of enteric viruses have been reported, organic flocculation still suffers from some limitations. The low pH used in this method may compromise the viability of some viruses, especially rotaviruses (Lewis and Metcalf 1988). In addition, the residual of beef extract remaining after organic flocculation may interfere with molecular detection methods (Schwab et al., 1995). Polyethylene glycol (PEG) precipitation offers an alternative for improved secondary concentration of viruses from the eluant (Lewis and Metcalf 1988). PEG is a non-toxic, chemically inert, water-soluble polymer that can be used to precipitate a wide variety of proteins. This method has been demonstrated to be reliable for partial purification of viruses in the eluant prior to the nucleic acid extraction and is thus, more compatible with the RT-PCR assay (Schwab et al., 1995; Huang et al., 2000).

Centrifugal ultrafiltration has been used in conjunction with tangential flow filtration (Jiang et al., 2001) or membrane adsorption-elution techniques (Katayama et al., 2002) as a secondary concentration for enteric viruses in water samples. Examples of commercially available centrifugal ultrafiltration device used to concentrate viruses are Amicon Ultra and Centriprep centrifugal filter units from Millipore with molecular weight cut-off of 30 000 to 100 000. Centrifugal ultrafiltration do not require manipulation of water samples and have shown a mean recovery of 74% for poliovirus seeded in ultrapure water (Haramoto et al., 2004).

2.5 Detection and quantification methods for enteric viruses

The selection of detection method for enteric viruses in the aquatic environment largely depends on whether the target viruses are cultivable. Detection of cultivable enteroviruses, for example by viral replication in cell culture, demonstrates the infectivity of isolated viruses which is of major relevance from the public health point of view. For the detection of non-cultivable viruses such as noroviruses, molecular approaches targeting nucleic acids, which do not give a direct indication of infectivity, must be used. The combination of cell culture with molecular methods and flow cytometry has also been developed to detect enteric viruses which show no evidence of infectivity in any other way. These main approaches for the detection, enumeration and quantification of enteric viruses in water are discussed in the following sub-sections.

2.5.1 Detection by cell culture

To date, the standard detection method for viruses is the cell culture assay (USEPA 1984). In this cultivation assay, viruses are counted as plaques in solid monolayers of cells, as tissue culture for 50% infective dose (TCID₅₀) or most probable number (MPN) in liquid suspensions (Payment 2001).

Detection of enteroviruses by cell culture is relatively well established since all known enteroviruses can be propagated in animal and human tissue cultures (Pallansch and Roos 2001). Dahling and Wright (1986) conducted extensive studies to optimize the buffalo green monkey (BGM) kidney cell line for monitoring enteroviruses in environmental samples and made recommendations in respect of various assay parameters for standardizing cell culture procedures. In a comparative study with BGM kidney cells and nine other cell types, the authors found that the BGM cell line was more sensitive for isolating waterborne enteroviruses. Total culturable viral assay (TCVA) by using the BGM cell line is the standard method for the enumeration of viruses from water (USEPA 1995). However, a recent study by Rodriguez et al. (2008) demonstrated that the primary liver carcinoma

(PLC/PRF/5) cell line was more sensitive than the BGM cell line for the detection of cultivable viruses (adenoviruses and enteroviruses) in treated sewage.

Adenoviruses do not form plaques but can be cultivated in human cell culture such as primary human embryo kidney (HEK) cells (Krisher and Menegus 1987). More recently, a plaque assay for fastidious human adenovirus serotypes 40 and 41 by using A549 cells (an epithelial lung carcinoma cell lines) has been developed (Cromeans et al., 2008). However, the application of this plaque assay for the recovery of wild type adenoviruses from environmental samples has not been demonstrated. Human rotaviruses may be adapted to grow in monkey kidney cells (Birch et al., 1983). Both continuous cell lines and primary cells of monkey kidney have been evaluated, but greater success and reproducibility were reported with the latter (Hasegawa et al., 1982; Wyatt et al., 1983; Ward et al., 1984). In the later study, however, Kitamoto et al. (1991) demonstrated that some human rotavirus strains replicated more effectively in human colonic carcinoma (CaCo-2) cell line than in monkey kidney cells. The authors suggested that the CaCo-2 cell line may be useful in general laboratories of rotavirus research.

Another important milestone in viral propagation was achieved when Lee and Kurtz (1981) demonstrated the isolation of human astrovirus in primary human embryo kidney (HEK) cells, a continuous line of rhesus monkey kidney cells (LLCMK2) and primary baboon kidney (PBK) cells. However, attempts to cultivate human astrovirus in other cell lines were not successful. Pinto et al. (1994) reported the isolation of astrovirus, rotavirus, reovirus, adenoviruses and enteroviruses (poliovirus 1, coxsackievirus A24, enterovirus 70) using the CaCo-2 cell line, after treatment with trypsin. These authors demonstrated that nucleic acids from infectious rotavirus, astrovirus, and adenovirus type 40 could be amplified after inoculation of CaCo-2 cells with sensitivity of 10^2 , 10^6 and 10^3 particles, respectively. The ability to achieve higher viral titers in CaCo-2 cells facilitates future studies of these enteric viruses isolated from the environment in cell culture (Pinto et al., 1995).

Hepatitis A viruses are usually difficult to adapt and grow in cell culture and have few or no visible effects on the host cell. Binn et al. (1984) examined seven different primate cell types for the growth of hepatitis A viruses and found that optimal replication often results from a particular cell type and temperature, however, no cytopathic effects were observed in all the isolates. In addition, attempts of Morace et al. (2002) to demonstrate the infectivity of the hepatitis A viruses present in sewage, by cultivation in cell culture, were unsuccessful. Noroviruses do not multiply in conventional cell culture or in animal models (Duizer et al., 2004). An *in vitro* cell culture assay by using 3-dimensional aggregates of a highly differentiated intestinal epithelial cell line has recently been described for human noroviruses (Straub et al., 2007). However, this complicated assay requires special cell culture techniques and the cell line is not widely available, hence making it difficult to use on a regular basis. Nonetheless, the development of a basis cell culture system for noroviruses has always been a primary goal in order to better assess the infectivity of noroviruses during water treatment or under various environmental stresses (Karim et al., 2004).

The major drawback of the viral cell culture assay is that it is laborious and time-consuming for conclusive results. This makes conventional cell culture an impractical method for routine monitoring of enteric viruses in environmental waters. In addition, conventional cell culture is susceptible to cytotoxic compounds in the environmental sample which may inactivate the cell monolayer, prior to virus infection and thus, leading to false-positive results.

2.5.2 Detection by flow cytometry

Flow cytometric protocols have been developed to detect different types of viruses in natural water. Marie et al. (1999) and Brussaard et al. (2000) have enumerated viruses in aquatic systems by flow cytometry after staining with the nucleic acid-specific dye, SYBR Green I. However, the flow cytometric protocols developed thus far are primarily used to detect marine viruses and bacteriophages (Brussaard 2004). In addition, Brussaard et al. (2000) found that the fluorescence

signals of flow cytometry for small genome-sized RNA viruses (7.4 – 14.5 kb) were just below the limit of detection. Thus, it may not be possible to detect most waterborne enteric viruses by flow cytometry with the current sensitivity of the instrument. A recent developed microsphere-based flow cytometric assay using antibody coated capture beads to isolate target viruses primarily in clinical samples offers an alternative to overcome the current limitation of flow cytometry (Samoylova and Smith 1999; Yan et al., 2004). Nevertheless, the application of this assay has not been evaluated with various environmental samples.

For specific enteric viruses, cell cultures coupled with flow cytometry have been developed to detect infectious rotaviruses in environmental, clinical, and food samples (Abad et al., 1997; Abad et al., 1998; Barardi et al., 1998; Barardi et al., 1999). This integrated method could be used to measure levels of occurrence, inactivation rates, and survival times for rotavirus and other enteric viruses in aquatic environments. However, only enteric viruses for which appropriate cell lines have been produced can be detected and quantified by this integrated method. A study by Qin et al. (2005) shows that fixation and permeabilization of the host cell membrane are critical to the success of this integrated method.

2.5.3 Detection by molecular techniques

Advances in molecular technologies offer an attractive alternative for improved and rapid detection of a diverse group of enteric viruses in water (Iqbal et al., 2000; Rose and Grimes 2001). Molecular techniques such as polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR (for the detection of RNA viruses) have emerged as sensitive and specific approaches for the detection of low levels of viral nucleic acids in various environmental waters (Toze 1999). PCR is an *in vitro* enzymatic amplification of specific genomic sequences followed by detection of the amplified nucleic acid by gel electrophoresis or probe hybridization. DNA microarray technology is a new and promising tool that allows the detection of diverse individual sequences in complex DNA samples simultaneously.

2.5.3.1 Qualitative PCR

Application of PCR for the detection of enteroviruses in different environmental samples generally has been extensively described (Touganidou and Botzenhart 1993; Gilgen et al., 1995; Muscillo et al., 1995; Shieh et al., 1997; Abbaszadegan et al., 1999; Schvoerer et al., 2001; Hot et al., 2003). This picornavirus group contains highly conserved nucleotide sequences at the 5' end of the genome (5' non-translated region), which are used to design pan-enterovirus primers for the PCR detection of all enterovirus species. Moreover, PCR has been extended to cover other enteric virus groups in the aquatic environment such as hepatitis E viruses (Grimm and Shay Fout 2002), adenoviruses (Puig et al., 1994; Formiga-Cruz et al., 2005), astroviruses (Marx et al., 1995; El-Senousy et al., 2007), rotaviruses (Green and Lewis 1999; van Zyl et al., 2004; Kittigul et al., 2005), and hepatitis A viruses (Tsai et al., 1994).

The application of molecular techniques and, in particular, the PCR has improved the detection of non-cultivable noroviruses in environmental samples (Loisy et al., 2000; Wyn-Jones et al., 2000; Fout et al., 2003; Haramoto et al., 2004; Lodder and Husman 2005; Ueki et al., 2005; La Rosa et al., 2007; Lee and Kim 2008a). Genetic diversity of noroviruses makes it difficult to develop a broadly reactive RT-PCR assay to detect all norovirus strains (Atmar and Estes 2001). The RT-PCR primers that target the viral RNA-dependent RNA polymerase (RdRp) gene in ORF 1 or capsid gene in ORF 2 have been designed to detect noroviruses GI and GII. (Jiang et al., 1999; Vinje et al., 2004; La Rosa et al., 2007). Recently, a newly designed nested RT-PCR assay for norovirus GIV has been reported, which may facilitate the detection of this rare norovirus genogroup in environmental samples (La Rosa et al., 2008).

2.5.3.2 Quantitative real-time PCR

The ability to measure the quantity of virus in an environmental sample is of paramount importance in determining the extent of pollution, and the effectiveness of the viral inactivation process. Quantitative real-time PCR (QPCR) is a relatively new molecular technique which enables quantification of DNA/cDNA targets by monitoring amplified products during cycling as indicated by increased fluorescence (Mackay et al., 2002; Walker 2002). QPCR was first developed for clinical diagnostics and has recently been adapted for environmental monitoring of enteric viruses because of higher sensitivity and specificity, and faster rate of detection.

A fluorescent dye such as SYBR Green I that intercalates with double-stranded DNA is the most common chemistry used in QPCR. However, SYBR Green I QPCR has not been widely used to detect and quantify enteric viruses in environmental waters (Laverick et al., 2004; Brooks et al., 2005). This could be due to the detection chemistry not being target sequence specific and thus, susceptible to false positive results especially in complex environmental matrices. Melting curve analyses are required as additional amplicons confirmation for the reaction using SYBR Green I chemistry. The dual-labeled fluorescent probe such as TaqMan probe has been the most popular technique of choice in environmental virology due to its specificity. The advantage of this assay is that the target amplicon is verified by the probe which recognizes internal amplicon sequences. This fluorochrome-tagged probe-based quantification technique has been used to detect astroviruses (Le Cann et al., 2004), adenoviruses (Choi and Jiang 2005; He and Jiang 2005; Van Heerden et al., 2005; Haramoto et al., 2007; Xagorarakis et al., 2007; Muscillo et al., 2008), enteroviruses (Fuhrman et al., 2005), hepatitis A viruses (Gersberg et al., 2006; Rose et al., 2006; De Paula et al., 2007) and noroviruses (Katayama et al., 2008; Hamza et al., 2009) in various environmental water samples.

2.5.3.3 Issues associated with PCR in environmental virology

One of the major limitations of PCR-based assays is the susceptibility to environmental inhibitors such as humic compounds, high divalent cation concentrations and salts (Wilson 1997). These substances can bind to magnesium ions and consequently prevent them from acting as cofactors for enzymes used in the amplification reaction. Lewis et al. (2000) demonstrated that humic acid at a concentration of 5 to 150 mg/L significantly reduced the RT-PCR efficiency in viral detection. Several techniques have been used to remove inhibitory substances from the sample, e.g. treatment of the sample with resins and chelators (Abbaszadegan et al., 1993) and simple dilution of the sample extract (Gregory et al., 2006).

In addition, there has been much debate over whether the PCR-based assay is a legitimate technique for measuring infectious viruses in the environment (Sobsey et al., 1998; Richards 1999; Nuanualsuwan and Cliver 2002). However, most waterborne enteric viruses are environmentally robust and naked RNA does not survive in the environment (Limsawat and Ohgaki 1997). Tsai et al. (1995) demonstrated that naked enterovirus RNA could not be detected by RT-PCR after two days of incubation at both 4°C and 23°C in unfiltered seawater. This suggests that naked RNA which is not protected by a protein coat is more easily degraded by RNases in the environmental water than intact virus. A study by Bhattacharya et al. (2004) which shows significant correlation between signals generated after RT-PCR amplification of viral genome and the presence of infectious viruses in the samples, supports the above-mentioned hypotheses. Moreover, several studies have observed the correlation between the reduction of viral infectivity and the persistence of viral genomes in surface waters, supporting the use of molecular techniques for the detection and quantification of viruses in water (Skraber et al., 2004; Wetz et al., 2004; Espinosa et al., 2008).

Alternatively, integration of cell culture with PCR has permitted the rapid and indirect detection of infectious viruses in the environment (Reynolds et al., 1996; Greening et al., 2002). By integrated cell culture (ICC)/strand-specific RT-

PCR, Jiang et al. (2004) reported detection of infectious hepatitis A viruses at an inoculation level of 10^0 TCID₅₀ per flask within 4 days compared with 2 to 4 weeks by immunological methods. The principle of this assay is the demonstration of viral replication in infected host cells by detecting the formation of the RNA replicative intermediate by RT-PCR. Ko et al. (2003) developed a messenger RNA (mRNA) real-time RT-PCR assay to detect infectious adenoviruses within 3 days by specifically targeting synthesized mRNA during replication in cell culture. However, the applicability of these novel approaches to real environmental samples is still uncertain.

2.5.3.4 DNA microarrays

DNA microarrays are glass microslides or nylon membranes containing a high density of immobilized nucleic acids in an ordered two-dimensional matrix (Lemarchand et al., 2004). This can be a short section of genes or other DNA elements that are used as probes to hybridize a cDNA sample. A current limitation of DNA microarrays is that they do not offer the low detection levels available by PCR and thus, this technology may not be suitable for the direct detection of low concentrations of enteric viruses in environmental matrices. Nevertheless, current microarray technology coupled with PCR or cell culture may be useful for monitoring genetic variability of enteric viruses for epidemiology and microbial source tracking studies (Wong et al., 2007). There is an increasing trend focused on developing and adapting microarrays for the detection and genotyping of enteric viruses in clinical samples (Chizhikov et al., 2002; Wang et al., 2002; Jaaskelainen and Maunula 2006; Brown et al., 2008). However, none of these microarray protocols have been applied to environmental water samples. More recently, Brinkman and Fout (2009) reported the development of microarray for genotyping noroviruses in seeded water samples after RT-PCR. Further study is needed to evaluate this method for the rapid identification of different norovirus genotypes in real environmental samples.

2.6 Occurrence of enteric viruses in aquatic environments

Enteric viruses may be introduced to aquatic environments through human activities such as urban stormwater runoff, agricultural runoff, leaking sewers and discharge of untreated and treated wastewaters. Human enteric viruses are shed in extremely high numbers in the fecal matter of infected individuals. Susceptible individuals suffering from gastroenteritis or hepatitis may excrete from 10^5 to 10^{11} viral particles per gram of stool (Farthing 1989). In addition, animal-specific enteric viruses are important sources for fecal contamination of environmental waters (Fong and Lipp 2005). Recent studies on the occurrence of human enteric viruses in various environmental water samples are summarised in Table 2.5.

2.6.1 Factors controlling the occurrence and survival of enteric viruses in water environments

Several authors have highlighted the potential impacts of climate variability on waterborne disease (Patz et al., 2000; Rose et al., 2001) and one of the most important factors may be due to rainfall events. For example, a retrospective long-term study in the United States has demonstrated a statistically significant association between rainfall events and waterborne disease outbreaks (Curriero et al., 2001). Several studies have also showed a correlation between the incidence of rainfall and the deterioration of microbial water quality (Ferguson et al., 1996; Griffin et al., 2000; Lipp et al., 2001).

Adsorption of viruses to sediments is believed to increase their persistence in aquatic environments by offering protection from various environmental stresses (LaBelle and Gerba 1982). In a comparative detection of enteric viruses in wastewaters and sediments by Green and Lewis (1999), no hepatitis A virus was detected in either raw sewage or final effluent, but was present in 7 out of 8 sediment samples in the immediate vicinity of a sewage outfall. In addition, Ferguson et al. (1996) detected enteric viruses in both urban estuary water and sediment samples during wet weather, but viruses persisted only in the sediment

during the dry season. These studies suggest that assessing virological quality of water related to public health risk should take into account the survival and accumulation of enteric viruses in sediments, as well as the likelihood of their resuspension and redistribution by natural disturbance.

Temperature is often reported as the most important environmental variable influencing the fate of enteric viruses in the aquatic environment (Bosch et al., 1997; Hernroth et al., 2002; Skrabber et al., 2004). In general, the survival of most enteric viruses and their indicators is prolonged at lower temperature, whereas viral inactivation occurs at elevated temperatures. Exposure of viruses to high temperatures can damage viral nucleic acids and capsid proteins, thereby affecting adsorption of viruses to the susceptible host cell and preventing viral replication. The effect of temperature on the survival of enteric viruses in natural waters was found to be virus type dependent (Enriquez et al., 1995; Nasser and Oman 1999).

Sunlight irradiation is another environmental factor responsible for the rapid inactivation of viruses in water environments. It has been suggested that two major pathways are involved in the sunlight inactivation of viruses in natural waters; photo-biological (DNA damage) and photochemical (capsid and host-binding proteins damage) (Sinton et al., 1999). Fujioka and Yoneyama (2002) showed that poliovirus, echovirus and coxsackievirus suspended in seawater were stable for six hours in the absence of sunlight but were inactivated at the same rate in the presence of sunlight. Adenoviruses have been demonstrated to be more resistant to UV irradiation than other virus groups, including coliphage MS2 (Meng and Gerba 1996; Gerba et al., 2002). It is believed that adenoviruses with double-stranded DNA are able to use the host cell enzymes to repair DNA damage caused by UV irradiation, with the undamaged DNA strand serving as a template (Thurston-Enriquez et al., 2003; Eischeid et al., 2009). A study by Nuanualsuwan et al. (2002) using feline calicivirus as a surrogate for human caliciviruses, showed that the feline calicivirus appeared to be the most UV resistant among the tested viruses (hepatitis A virus, poliovirus type 1, coliphages MS2 and phiX174).

Salinity has been found to have little direct influence on virus survival in seawater (Le Guyader et al., 1994). Salinity, however, has been demonstrated to increase viral aggregation in both artificial and natural seawaters, which contributes to loss of infectious viral particles (Gerba and Schaiberger 1975). A comparative study by Schaper et al. (2002) on the susceptibility of four genotypes of F-specific RNA coliphages to various environmental factors including salt concentration and pH in freshwater showed that the pattern of resistance to inactivation varied according to the genotype. The impact of extreme pH values on viral decay is considered less important as most enteric viruses can be quite resistant to low and high pH (Ferguson et al., 2003).

The grazing of pathogens by aquatic invertebrates has several implications for viral transport and distribution through natural waters (Brookes et al., 2004). Gordon and Toze (2003) compared the influence of several environmental conditions such as temperature, oxygen, nutrient levels and presence/absence of microorganisms on the survival of poliovirus and coxsackievirus in groundwater. The authors suggested that the most influential factor affecting the decay of viruses is the presence of indigenous groundwater microorganisms. However, this biotic factor was not directly studied and therefore the exact mechanism behind this inactivation is uncertain.

2.6.2 Occurrence of enteric viruses in untreated and treated wastewaters

Many enteric viruses are expected to be found in sewage, although their numbers will vary according to the epidemiological status of the populations. Environmental surveillance, mostly sewage monitoring, has been used to assess the circulation of various human enteric viruses in different geographical regions (Sedmak et al., 2003; Myrmel et al., 2006). Moreover, sewage surveillance for enteroviruses has been proved to be useful for monitoring the effectiveness of the poliovirus eradication program and the introduction of wild-type poliovirus strains into communities (Poyry et al., 1988; Deshpande et al., 2003; Pavlov et al., 2005; Hovi et al., 2007).

The enterovirus was the first enteric virus group isolated in sewage, in the 1940s, and they continue to be among the most widely studied (Metcalf et al., 1995). Their apparent higher prevalence may be associated with available cell lines for their isolation and propagation. The occurrence of enteroviruses in wastewaters has been shown to follow a similar pattern in temperate countries, across Europe, with the coxsackievirus B as the most prevalent serotypes (Hovi et al., 1996; Papaventsis et al., 2005; Costan-Longares et al., 2008; Petrinca et al., 2009). In tropical regions, Dahling et al. (1989) reported the prevalence of coxsackievirus B5 in wastewaters of Puerto Rican communities, with virus concentration exceeded 10^5 PFU/liter and 10^4 PFU/liter in sewage and secondary effluent, respectively. Although enteroviruses have served as the primary enteric virus indicator in previous studies, several studies have demonstrated that adenoviruses are more abundant in sewage (Pina et al., 1998; Vantarakis and Papapetropoulou 1999; Myrmel et al., 2006; Katayama et al., 2008; Symonds et al., 2009). However, a recent study by Petrinca et al. (2009) reported that adenoviruses were less prevalent than enteroviruses in sewage and secondary effluent.

Hepatitis A virus will be present in sewage if the virus is circulating within the community especially in regions with a high level of endemic disease such as India (Vaidya et al., 2002) and Brazil (Villar et al., 2007). However, Hepatitis A viruses have also been detected in the urban sewage of geographical areas presently considered as non- or low endemic areas (Pina et al., 2001; Petrinca et al., 2009). Although rotaviruses are the main cause of infantile gastroenteritis worldwide (Parashar et al., 2006), there are only limited numbers of reports on the presence of rotaviruses in wastewaters. Nevertheless, findings from several studies have suggested that monitoring of sewage may provide an overview of the epidemiology of rotaviruses circulating in the community (Baggi and Peduzzi 2000; Villena et al., 2003; Meleg et al., 2008), with important implications for vaccine development.

There are only a few studies characterizing astroviruses and noroviruses isolated from sewage samples by DNA sequencing and phylogenetic analysis. Nadan et al. (2003) reported the multiple genotypes of astroviruses in sewage

samples which were closely related to the clinical isolates. In contrast, Meleg et al. (2006) and Rodriguez-Diaz et al. (2009) demonstrated that the majority of astroviruses isolated from sewage samples belonged to genotype 1 and genotype 8, respectively. A great genetic diversity of noroviruses in raw and treated sewage has been described (van den Berg et al., 2005; Iwai et al., 2009), suggesting that human exposure to multiple norovirus strains in sewage contaminated water may lead to the occurrence of new norovirus recombinants.

The efficiency of wastewater treatment processes for reducing and inactivating enteric viruses has been evaluated by monitoring their presence directly in treated wastewater samples (El-Senousy et al., 2007; Nordgren et al., 2009). Thompson et al. (2003) reported the isolation of infectious adenoviruses in 15 out of 16 UV-disinfected secondary- and tertiary-treated wastewater samples. This finding suggests that UV doses effective at meeting certain wastewater regulations for total coliform bacteria may not provide adequate inactivation of the human adenoviruses.

2.6.3 Occurrence of enteric viruses in surface waters

More information is available on the occurrence of enteroviruses in surface waters compared with other enteric virus groups (Table 2.5). Infectious enteroviruses have been detected in most water matrices such as freshwaters (Murrin and Slade 1997; Schvoerer et al., 2001) and coastal waters (Reynolds et al., 1995; Pallin et al., 1997). A high frequency of the detection of enterovirus genomes in surface water (88%) compared with infectious enteroviruses (3%) was observed by Hot et al. (2003). The authors suggested that the detection of enterovirus genomes may be useful for predicting the occurrence of waterborne enteric viruses. Recent studies in Taiwan reported the prevalence of major epidemic genotypes of enteroviruses in surface waters used for drinking water supplies (Chen et al., 2008b; Hsu et al., 2009) and recreational activities (Chen et al., 2008a; Hsu et al., 2008), highlighting the importance of routine monitoring of pathogenic viruses in drinking and recreational water sources.

Early detection of human adenoviruses in water environments is achieved by using conventional cell cultures (Tani et al., 1992; Grohmann et al., 1993; Tani et al., 1995). In a later study of the presence of enteric viruses in various environmental water samples, Pina et al. (1998) noted that human adenoviruses were most frequently detected throughout the year, and suggested that the PCR detection of adenoviruses may be useful as an index of the presence of human viruses in the environment. Other studies have also shown the high prevalence of adenoviruses in surface waters compared with other enteric virus groups (Lee and Kim 2008b; Hamza et al., 2009). In South Korea, Lee et al. (2004) reported the detection of infectious adenoviruses in 72.5% of the 40 river water samples by integrated cell culture-PCR. This is in contrast with other studies which showed that adenovirus-positive samples by PCR were negative by cell cultures or integrated cell culture-PCR (Greening et al., 2002; Choi and Jiang 2005). By using DNA sequencing, most studies have identified enteric serotypes 40 and 41 as dominant adenovirus serotypes in water environments (Santos et al., 2004; Pusch et al., 2005; Van Heerden et al., 2005; Albinana-Gimenez et al., 2006). Nevertheless, only a few studies reported the occurrence of non-enteric adenoviruses in surface waters (Lee et al., 2004; Muscillo et al., 2008).

Among the enteric viruses of public health concern, the occurrence of astroviruses in surface waters has not been widely studied (Marx et al., 1998; Chapron et al., 2000; Taylor et al., 2001; Pusch et al., 2005; Miagostovich et al., 2008; Rodriguez-Diaz et al., 2009). In a one-year field survey for astroviruses and hepatitis A viruses in the Klip River and Vaal Dam, South Africa, Taylor et al. (2001) highlighted that infectious human astroviruses and hepatitis A viruses were detected in both water matrices, with a seasonal pattern noted for hepatitis A viruses but not for astroviruses. However, no correlation was made between the seasonal occurrence of hepatitis A viruses and the overall incidence of hepatitis A in the community. In contrast, the seasonal variation and clinical epidemiology of human astroviruses were noted in wastewater samples from Southern France and Barcelona after a two-year field study (Pintó et al., 2001). A report of Sedmak et al. (2005) on the occurrence of culturable viruses in lake water samples and sewage samples from

August 1994 through July 2003 shows significant seasonal fluctuation of infectious viruses in Milwaukee, Wisconsin. In Japan, Haramoto et al. (2005) reported the seasonal variation of norovirus concentrations in river water samples during a one-year survey, with higher concentrations in winter. Whether the seasonal occurrence of human enteric viruses in the environment is constrained by virus types, geographical distributions and water matrices is still uncertain.

The number of reports on the occurrence of noroviruses in surface waters escalated over recent years owing to the advent of molecular techniques and the increased awareness of this emerging waterborne pathogen (Table 2.5). Lodder and Husman (2005) surveyed waters of the river Waal and Maas in The Netherlands and found that noroviruses were present at 4 to 4,900 PCR-detectable units (PDU) per liter of river water. Phylogenetic analysis conducted by the authors showed the presence of seven different genotypes of noroviruses in river water samples, with GII/4 Lordsdale as the most prevalent strain in the one-year sampling period. The authors attributed the presence of noroviruses in the Waal and the Maas to the discharge of both raw and treated sewage into the rivers. A recent occurrence study in South Korea revealed that the detection rates of norovirus GI were higher than those of norovirus GII in river waters (Lee and Kim 2008a). The authors also observed a great genetic diversity of human noroviruses in surface waters.

There is increasing evidence which shows that bacterial indicators are inadequate for assessing the viral contamination of environmental waters (Wyer et al., 1995; Vantarakis and Papapetropoulou 1998; Noble and Fuhrman 2001; Denis-Mize et al., 2004; Horman et al., 2004; Ballester et al., 2005; Pusch et al., 2005; Miagostovich et al., 2008; Hernandez-Morga et al., 2009; Wong et al., 2009). In a survey of human enteric viruses in canal waters throughout the Florida Keys using RT-PCR, Griffin et al. (1999) found 79%, 63%, and 10% of the sites were positive for the presence of enteroviruses, hepatitis A viruses, and noroviruses, respectively. The study in Florida Keys shows that: (i) viral contamination occurred despite the acceptable occurrence levels of bacterial indicators as suggested by USEPA; and (ii) wastewater discharge was the major cause of viral contamination in the canals

and near-shore waters of the Florida Keys. In a survey of adenoviruses in coastal waters of Southern California, Jiang et al. (2001) found that 4 of the 12 samples were positive for human adenoviruses by nested PCR, with the most probable number of adenoviral genomes ranging from 880 to 7,500 per liter of water. The authors noted that the presence of human adenoviruses did not correlate with the bacterial indicators throughout the field study, especially when no bacterial indicators exceeded the daily threshold in coastal waters. In the later survey at the same location and the Newport Bay watershed, the authors observed the same phenomena (Jiang and Chu 2004; Jiang et al., 2007).

There are only a few studies on the occurrence of enteric viruses in water environments in tropical regions, especially in Southeast Asia (Table 2.5). A field survey by Kittigul et al. (2005) in Thailand reported the detection of rotavirus RNA in river and canal water samples. The authors found that a majority of the rotavirus sequences isolated was closely related to the rotavirus G9. A follow-up occurrence study in Thailand showed that hepatitis A viruses were less prevalent than rotaviruses in surface waters (Kittigul et al., 2006). A study in Indonesia showed that hepatitis A viruses and adenoviruses were prevalent in river water samples and floodwaters (Phanuwan et al., 2006), suggesting a higher risk of viral infections through the contamination of drinking water sources and direct contact with floodwaters during flooding events.

2.6.4 Occurrence of enteric viruses in groundwater and drinking water

Human enteric viruses can contaminate groundwater and are responsible for a significant proportion of infectious gastroenteritis related to groundwater consumption as shown in Table 2.3. Several studies have systematically monitored groundwater sources of drinking water for the occurrence of enteric viruses. In the United States, Abbaszadegan et al. (2003) investigated the occurrence of human viruses in groundwater samples collected from 448 sites in 35 states. The authors demonstrated that 31.5% of the sampling sites were positive for at least one virus group by RT-PCR. Borchardt et al. (2003) reported that 8% of the 50 private

household wells in Wisconsin were positive for enteric viruses. The authors noted that the viral contamination appeared to be transient since none of the sequential well water samples were positive for viruses. In a field survey for enteric viruses in municipal drinking water wells by the same authors, all samples were negative for conventional microbial indicators for water quality, namely coliphages, total coliform bacteria, *Escherichia coli*, and fecal enterococci even though enteric viruses were detected by RT-PCR (Borchardt et al., 2004). This finding is in agreement with the later occurrence study in Canada (Locas et al., 2007), suggesting the limitation of these microorganisms in predicting the occurrence of human enteric viruses in groundwater.

Evidence has been published on the presence of human enteric viruses in finished drinking water and tap water, highlighting that conventional drinking water treatment processes may have a limited capacity for virus removal. The occurrence of enteroviruses and adenoviruses in drinking water has been widely reported in South Africa (Grabow et al., 2001; Van Heerden et al., 2003; Van Heerden et al., 2004; Vivier et al., 2004; Ehlers et al., 2005; Van Heerden et al., 2005) and South Korea (Cho et al., 2000; Lee and Kim 2002; Lee and Jeong 2004; Lee et al., 2005). Using conventional cell culture, Ali et al. (2004) provided the evidence of the presence of infectious enteroviruses in water samples after sand filtration at concentrations ranging from 10 to 33.3 PFU/liter. In a recent study, Gutierrez et al. (2007) demonstrated the presence of proteins of rotavirus and norovirus in treated drinking water by using the enzyme immunoassay method. The authors suggested the detection of viral proteins may indicate the presence of infectious viruses in water samples.

Table 2.5 Recent studies on the occurrence of enteric viruses in water environments

Location	Sample type (volume)	Concentration method	Detection method	Virus isolated	Findings	Significance of the study	Reference
Southern California, United States	Urban rivers and creeks	Vortex flow filtration (VFF) or Centriprep concentrator (Millipore) with 100kD membrane	RT-PCR probe hybridization, nested PCR for AdV	AdV, EV, HAV	52%, 62% and 76% of the sites were positive for AdV, EV and HAV, respectively.	- First direct evidence that human viruses are prevalent in southern California urban rivers; - Urban run-off impacts coastal water quality most significantly during storm season.	Jiang and Chu (2004)
San Diego, United States	Coastal water after rain events > 0.5 cm (4 L)	Adsorption-elution (negatively charged membrane, NaOH), Centriprep Concentrator	RT-PCR, sequencing, QPCR (SYBR Green)	HAV	- 75% (6/8) positive for HAV by RT-PCR; - All positive by QPCR ($90\text{--}3.5 \times 10^3$ copies/L).	- The presence of inhibitors affected QPCR and dilution of cDNA enabled successful amplification; - No correlation was observed between the fecal coliforms and HAV concentration.	Brooks et al. (2005)
Michigan, United States	Freshwater from recreational beaches (250-350 L)	Adsorption-elution (1MDS filter, beef extract), organic flocculation	QPCR (TaqMan), integrated cell culture-PCR	AdV	- 24% (14/58) tested positive for AdV; - Concentrations ranged from 7 to 3.8×10^3 particles/L; - Approx. 60% of ICC-PCR results agreed with the QPCR results.	- The occurrence of F species AdV in recreational beaches poses potential health risks especially to children.	Xagorarakis et al. (2007)

Table 2.5 (Continued)

Location	Sample type (volume)	Concentration method	Detection method	Virus isolated	Findings	Significance of the study	Reference
United States	Sewage and final effluent (10 ml)	Centriplus YM-50 and Microcon Ultracel YM-30 centrifugal concentration devices (Millipore)	Nested PCR and RT-PCR	EV, AdV, NV, RV, picobirnaviruses	- AdV and picobirnaviruses were found in 100% of sewage; - EV and NV were detected in 75% (9/12) and 58% (7/12) of sewage, respectively.	- This study uncovered previously unknown sequence diversity in human picobirnaviruses; - AdV and picobirnaviruses were proposed as potential marker of fecal contamination.	Symonds et al. (2009)
Venezuela	Sewage and sewage-polluted river (35 ml)	Ultracentrifugation	Nested PCR and RT-PCR, sequencing	EV, AstV, NV, AdV, RV	- Detection rates of all viruses $\geq 50\%$; - Sewage, EV and NV prevalent, 75% (9/12), River, AstV prevalent, 89% (16/18); - G1 type of RV, NV GII and AstV type 8 were identified.	- Correlation was observed between the RV strains in environmental samples and epidemiological data; - Impacts of raw sewage disposal into surface waters in South America.	Rodriguez-Diaz et al. (2009)
Mexico	Estuarine water (20 L)	Ultrafiltration (hollow fiber filter), PEG precipitation	Nested RT-PCR, sequencing	HAV, NV	- HAV was found in 80% (32/40) of samples, NV 70% (28/40); - HAV genotype IB and NV GII were predominant.	- No significant correlation was observed between bacterial indicator and viruses; - A potential novel NV GII genotype was identified.	Hernandez-Morga et al. (2009)

Table 2.5 (Continued)

Location	Sample type (volume)	Concentration method	Detection method	Virus isolated	Findings	Significance of the study	Reference
South Africa	-Sewage (1-2 L), surface water (1 L);	PEG precipitation, Ultrafree 15 centrifugal filter device (Millipore)	Integrated cell culture-RT-PCR, probe hybridization, sequencing	AstV	- AstV was detected in 87% (13/15) of sewage; - AstV-1 (dominant) to 5, type 7 and type 8 were identified.	- Genetic diversity of human AstV was first described in the environmental sample; - Correlation was observed between the clinical and environmental AstV isolates.	Nadan et al. (2003)
Barcelona, Spain	Sewage (40 ml), river and seawater (50 L)	-Sewage-ultracentrifugation; -Surface water-Adsorption-elution (positively charged membrane, glycine), ultracentrifugation	Nested PCR and RT-PCR	EV, AdV, HAV	- 93% (14/15), 40% (6/15) and 27% (4/15) of sewage samples were positive for AdV, EV and HAV, respectively.	- High detection rate for AdV by PCR suggests its application as an index of the presence of human viruses in the environment.	Pina et al. (1998)
Barcelona, Spain	Sewage (40 ml); river (250 L)	- Sewage, ultracentrifugation - River water, adsorption-elution (positively charge membrane, glycine), organic flocculation with beef extract, ultracentrifugation	RT-PCR, sequencing	HAV	- HAV was detected in 57.4% of sewage, 20-39.2% of rivers; - 95% prevalence of genotype I; - genotype IIIA was detected in 5% of sewage.	- Correlation was observed between the clinical and environmental HAV isolates; - Circulation of several HAV strains closely related to strains isolated worldwide.	Pina et al. (2001)

Table 2.5 (Continued)

Location	Sample type (volume)	Concentration method	Detection method	Virus isolated	Findings	Significance of the study	Reference
France	Sewage, treated effluent (1 L)	Ultracentrifugation	QPCR (TaqMan)	AstV	- AstV was detected in all influent (mean value 4.1×10^6 genomes/100ml) and effluent (mean value 1.0×10^4 genomes/100ml).	- A QPCR protocol based on 3' - end of the AstV genome; - No significant seasonal differences were observed for AstV in wastewaters.	Le Cann et al. (2004)
The Netherlands	Sewage (10 L), treated effluent (200-400 L), rivers (200-600 L)	Adsorption-elution (negatively charge membrane), PEG precipitation, spin column gel chromatography, Centricon 100	RT-PCR, probe hybridization	EV, ReV, NV, RV	- NVs were present at 4-4,900 PCR-detectable units (PDU)/L of river water; - GII/4 prevalent; - Other target viruses were also detected.	- Very divergent strains of NV were isolated from surface and urban sewage waters.	Lodder and Husman (2005)
The Netherlands	Sewage (650 ml), treated effluent (10 L)	Adsorption-elution (negatively charge membrane), PEG precipitation, spin column gel chromatography, Centricon 100	RT-PCR, sequencing	NV	- Average concentration of NV in sewage was 10^5 PDU/L; 10^3 PDU/L in treated effluent; - 11 different NV variants were identified (GI/6, GII/2 and GII/b were prevalent).	- The presence of multiple NV strains in wastewaters.	van den Berg et al. (2005)

Table 2.5 (Continued)

Location	Sample type (volume)	Concentration method	Detection method	Virus isolated	Findings	Significance of the study	Reference
Germany	Sewage (50 ml), treated effluent (5 L), lakeshore (10 L)	Flocculation with aluminium sulfate, ultracentrifugation	Nested PCR and RT-PCR, sequencing, QPCR (TaqMan)	EV, AdV, AstV, NV, HAV, RV	- High detection rates for EV, AstV and NV in all sample types; - AdV was not detected in surface waters.	- Surface water downstream of the sewage treatment plant may be a source for enteric viral infections though bacteriological parameters indicate an acceptable microbiological water quality.	Pusch et al. (2005)
Hungary	Sewage (1 L)	PEG precipitation	RT-PCR, sequencing	AstV	- 43% (15/35) of samples positive for AstV; - Genotypes 1 and 2 were identified.	- Prevalence of human AstV genotype 1 in sewage.	Meleg et al. (2006)
Rome, Italy	River, estuarine water, seawater (10 L), sewage (50 ml)	TFF, PEG precipitation, ultracentrifugation	Nested RT-PCR, sequencing	NV	- 12% (3/26) of seawater and all sewage samples positive for NV; - GI/4 dominant; - NV GI was detected only in sewage (GI/1, GI/2 and GI/4).	- A new broad-range nested RT-PCR targeting RdRp, amplifying fragments of 687 bp (GI) and 516 bp (GII); - Correlation was observed between the clinical and environmental NV isolates.	La Rosa et al. (2007)

Table 2.5 (Continued)

Location	Sample type (volume)	Concentration method	Detection method	Virus isolated	Findings	Significance of the study	Reference
Germany	River water (10 L)	Adsorption-elution (negatively charged membrane, high-salt alkaline phosphate buffer), PEG precipitation	QPCR (TaqMan, SYBR Green), sequencing	EV, AdV, RV, NV, polyoma-virus	- Detection rates 97.5% for AdV and polyomavirus, 90% for RV, 31.7% for NV - GII, one sample positive for EV; - Virus concentration ranged from $9.4 - 2.3 \times 10^4$ gene copies/L.	- Sequence analyses revealed the AdV and RV PCR could cross-react with animal viruses; - This study suggested the detection of human polyomavirus is the most specific marker for contamination of water with human sewage.	Hamza et al. (2009)
Japan	Sewage (100ml), secondary effluent, effluent after chlorination (1 L)	Adsorption-elution (negatively charged membrane and NaOH), Centriprep YM-50	QPCR (TaqMan)	EV, AdV, NV	- Summer, geometric mean value for NV GI in influent was 4.9 RT-PCR units/ml; NV GII was 9.1 RT-PCR units/ml; - Winter, geometric mean value for NV GI in influent was 190 RT-PCR units/ml; NV GII was 200 RT-PCR units/ml; - EV and AdV in influent, 17 and 320 RT-PCR units/ml, respectively.	- NVs were abundant in winter, no seasonal differences were observed for EV and AdV; - AdV was detected at the highest concentration throughout the year, preferred indicator of viral contamination.	Katayama et al. (2008)

Table 2.5 (Continued)

Location	Sample type (volume)	Concentration method	Detection method	Virus isolated	Findings	Significance of the study	Reference
Thailand	Canal and river (1 L), Sewage (1 L), Tap water (5 L)	Adsorption-elution (negatively charged membrane and tryptose phosphate buffer with glycine)	Nested RT-PCR, sequencing	RV	- RV was found in 16.7% (20/120) of water samples; - No RV was detected in tap water; - Genotyping: type G9.	- Fecal coliforms including <i>E. coli</i> were found in all RV-positive samples.	Kittigul et al (2005)
Thailand	Swamp, canal and river (1 L), Tap water (5 L)	Adsorption-elution (negatively charged membrane and tryptose phosphate buffer with glycine)	Nested RT-PCR, sequencing	HAV	- HAV was found in 15% (6/40) of swamp and 10% (3/30) of canal; - No HAV was detected in river and tap water; - HAV genotype IA.	- The presence of HAV in water is a potential health risk for the low socio-economic communities.	Kittigul et al (2006)
Korea	River (2 L)	Adsorption-elution (negatively charged membrane, beef extract), PEG precipitation	Nested RT-PCR, sequencing	NV	- 55.2% (32/58) and 44.8% (26/58) showed positive for GI and GII, respectively; - 8 GI genotypes and 7 GII genotypes were identified; - Major types were GI/1, GI/13 and GII/15.	- Multiple genotypes of NV were identified in environmental water samples; - Most of the strains were closely related to the Asian isolates.	Lee and Kim (2008a)

2.7 Summary of literature review

As discussed earlier, studies conducted in the 1940s with the propagation of polioviruses in cell culture demonstrated that enteric viruses could survive in sewage and be transmitted to receiving waters, from where enteric viruses could be recovered in an infectious state. Since that time, a growing number of enteric viruses responsible for gastroenteritis and hepatitis have replaced enteroviruses as the main target for detection in the water environment. Detection and enumeration of enteric viruses has undergone a significant transition from conventional cell culture to novel molecular techniques such as PCR-based assay with higher sensitivity and specificity. Detection of enteric viruses by PCR does not necessarily indicate their infectivity, but it is an excellent exploratory tool in environmental virology. Moreover, the use of PCR methods for the analysis of non-cultivable noroviruses and slow growing hepatitis A virus has been fundamental to our understanding of the occurrence of these human pathogens in water environments.

Virological examination of water samples is a complex task with multi-stage processes involving concentration of viruses in the sample followed by detection and confirmation steps. Sampling strategy for viruses in environmental waters particularly some concentration methods, can be remarkably ineffective. To date, there is no consensus on the most efficient approach for concentrating all enteric viruses in water samples. Thus, continued development and evaluation of methods for concentrating viruses in the water is of critical importance.

Enteric viruses consist of nucleic acid neatly wrapped up in a small protein capsid, which is exceptionally resistant to unfavorable environmental conditions, including those in water and wastewater treatment processes. From the literature cited in this chapter, it is clear that a growing number of pathogenic human viruses can readily be detected in different environmental waters being impacted by human sewage. Viral pathogens are prevalent throughout different water environments, even in developed countries. There has been a worldwide waterborne outbreak associated with emerging viruses, particularly noroviruses in the last few years,

causing problems with both drinking water and recreational water where mortality has been reported. However, the actual burden of waterborne infections caused by enteric viruses might have been underestimated because they are not routinely monitored both in clinical and environmental samples. Therefore, direct surveillance of water environments for enteric viruses is warranted to better protect public health.

A common trend among most cited occurrence studies is that the occurrence of common bacterial indicators such as *Escherichia coli* and coliforms, did not correlate significantly with the presence of enteric viruses in waters. Thus, the reliability of bacterial indicators for accurately reflecting viral contamination in environmental waters has been questioned. In addition, most occurrence studies are performed using only the positive-negative detection of enteric viruses and do not provide sufficient quantitative data on viral concentrations in natural waters. Further study is required to accumulate much more quantitative data on the occurrence levels of bacterial indicators, coliphages and enteric viruses simultaneously in different environmental waters particularly in the tropical region. Such information would be useful for understanding the fate of enteric viruses and their indicators in the aquatic environment. With advances in molecular technology, novel quantitative tools such as quantitative real-time PCR are available, which can speed up the analytical procedures.

Thus, an important objective of the present study is to provide qualitative and quantitative data on the occurrence and distribution of enteric viruses in the tropical aquatic environment. The molecular characterization of isolated viruses from the environment will be conducted in furthering our understanding of the true prevalence of individual strains in this region. This will improve our understanding of the occurrence of waterborne viruses in the tropical aquatic environment and thus, their potential impacts on public health.

Chapter 3

Materials and Methods

3.1 Enteric viruses

The positive control samples used in the detection of enteric viruses by molecular techniques were astrovirus serotype 1 (provided by Prof. A. Bosch, University of Barcelona), adenovirus type 40 (strain Dugan, ATCC 931), echovirus 11 (strain Gregory, ATCC VR-41) and hepatitis A virus (strain HM175/18f, ATCC VR-1402). Norovirus genogroup I (GI) and GII plasmids were provided by the DSO National Laboratories, Singapore.

3.1.1 Human astrovirus propagation in CaCo-2 cells

Astrovirus serotype 1 was propagated in human colonic carcinoma cells, CaCo-2 cells (ATCC HTB-37) growing in Eagle's minimal essential medium (MEM) supplemented with 0.03% glutamine, 0.075% sodium bicarbonate, 100 Units of penicillin/mL, 100 µg streptomycin/mL and 10% foetal bovine serum (FBS).

Astrovirus propagation was performed as previously described by Pinto et al. (1994). Briefly, confluent monolayers of CaCo-2 cells were incubated overnight in MEM without FBS (Figure 3.1). Since CaCo-2 cells are an adherent cell line, they grow flat and attach firmly on the tissue culture flask. Viral stocks were preactivated for 30 min with 10 µg/mL of trypsin at 37°C, serially diluted 10-fold, and inoculated onto cells. After a 60-min adsorption at 37°C, serum-free MEM with 5 µg/mL of trypsin was added. Plates were checked daily for cytopathic effect (CPE).

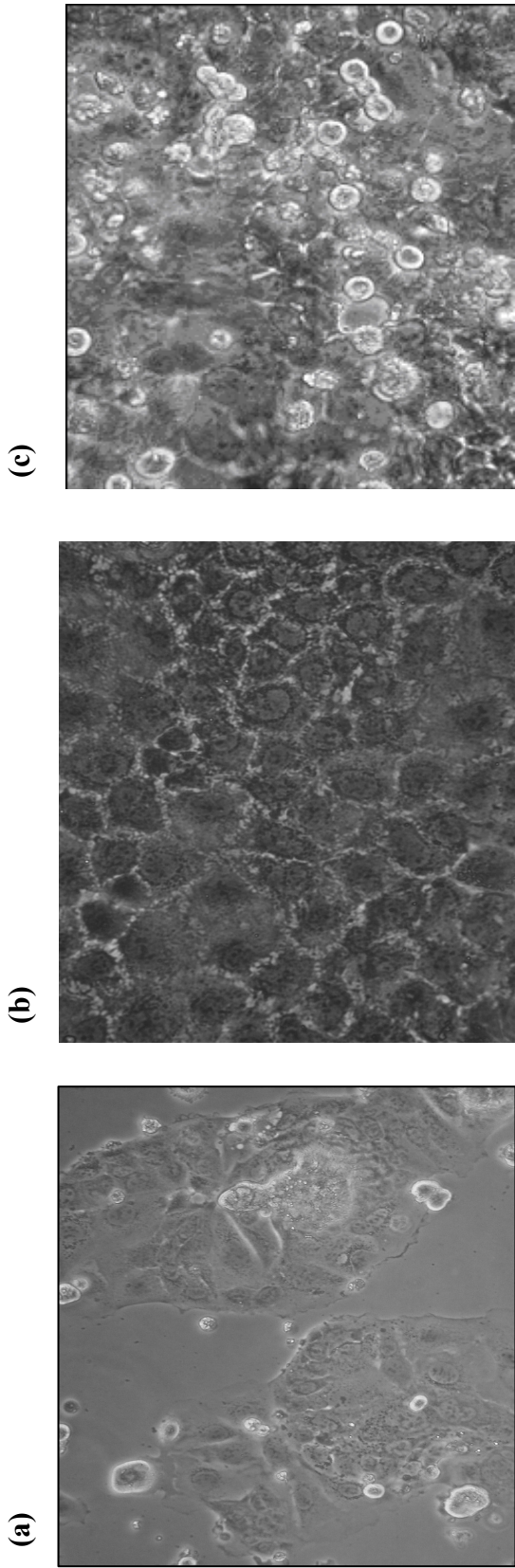


Figure 3.1 Human astrovirus propagation in CaCo-2 cells. (a) 1 day after subculture, (b) 5 days after subculture at approximately 80% confluence, (c) CPE in CaCo-2 cells produced by human astrovirus.

A distinct CPE that was characterized by the changes of cell morphology (rounding of the infected cells) was observed 3 days after infection of the human astrovirus. Some infected cells were detached from the tissue culture flask and floated in the medium (Figure 3.1c). Viruses were harvested after 3 to 5 days of post-infection by centrifugation and stored at -80°C.

3.2 Environmental water samples

3.2.1 Surface water samples from Marina Bay Catchment

Marina Bay is an estuarine bay located in the central of the city of Singapore, with a catchment area of 10 000 hectares. The bay is fed by discharges from the following waterways: Singapore River, Kallang River, Geylang River, Stamford Canal, Rochor Canal and Alexandra Canal (Figure 3.2). A barrage was completed in 2008 to turn Marina Bay into a confined freshwater reservoir. Marina Reservoir is the largest and most highly urbanized catchment in Singapore with a population of about 1 million and about 50 000 premises, including some of the oldest buildings in Singapore. The new reservoir provides new drinking water sources and a stable water body for various water-related recreational activities.

Between June 2006 to January 2009, surface water samples were collected from major rivers (upstream and downstream) and canals of Marina Bay (Figure 3.2). All sampling sites were located in highly urban settings. The bay which is fed by the discharges from these waterways was also sampled from March to June 2007. Ten-liter (rivers and canals) and 20-liter (estuarine bay) water samples were collected with autoclaved and sample-rinsed carboys and transported to the laboratory on ice for immediate processing.

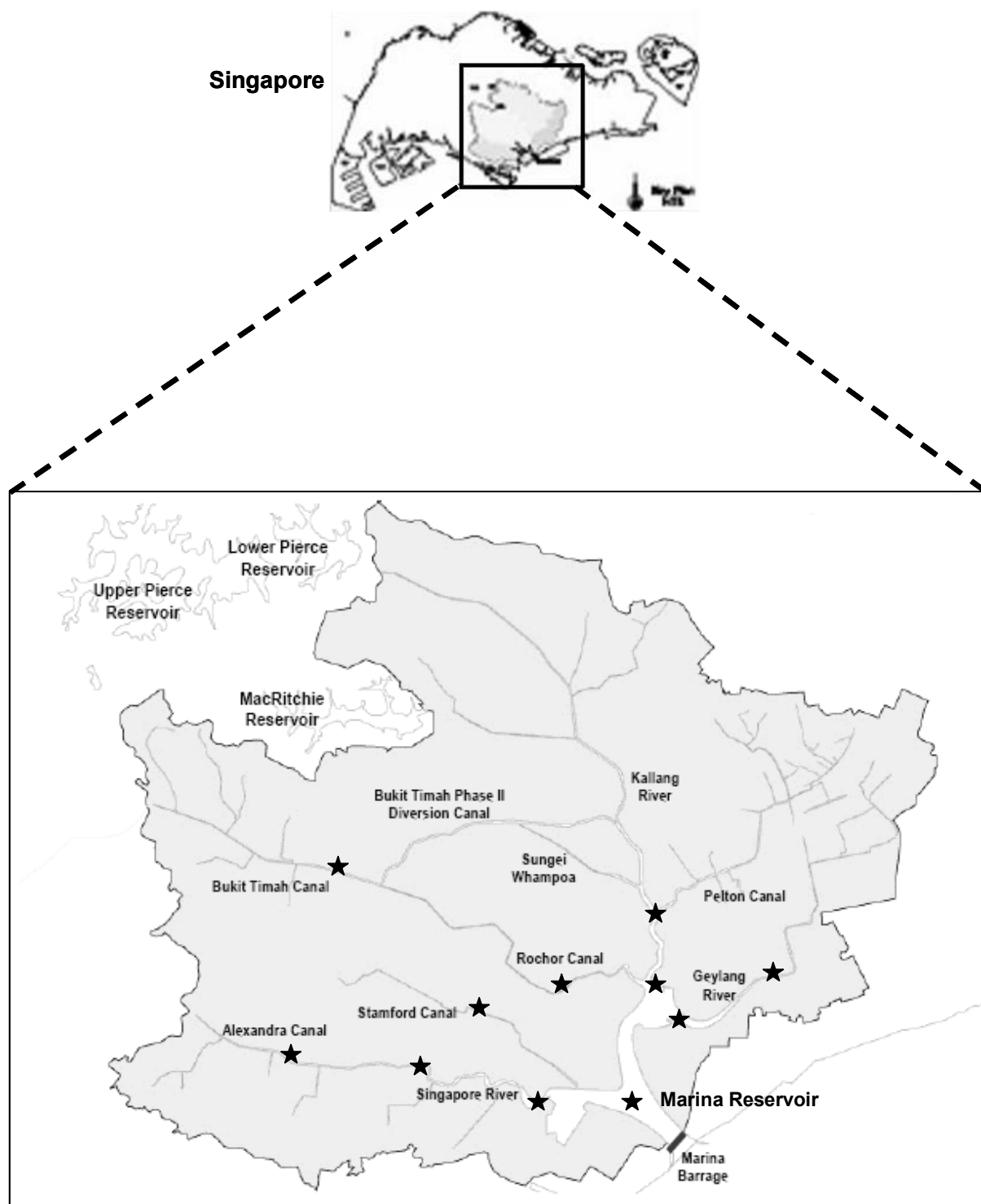


Figure 3.2 Map of Marina Bay and its tributary waterways showing sampling sites.

3.2.2 Wastewater samples

Sewage and secondary effluent samples were collected monthly from three water reclamation plants in Singapore from January to June 2007. Bedok Water Reclamation Plant (WRP), Kranji WRP and Ulu Pandan WRP receive wastewater from eastern, northern and western areas of Singapore, respectively. Wastewater is treated at the water reclamation plant using activated sludge processes and the secondary effluent is discharged into the ocean or further treated by membrane technologies and UV disinfection for reuse. One-liter of sewage and ten-liter of secondary effluent were collected and transported to the laboratory on ice for immediate processing.

3.3 Environmental water samples processing and concentration

The concentration procedures for surface water, sewage and secondary effluent samples are summarized in Figure 3.3.

3.3.1 Concentration of enteric viruses from urban sewage

Enteric viruses from one liter of sewage samples were recovered by polyethylene glycol (PEG) precipitation as previously described with modifications (Shieh et al., 1995). Briefly, sewage sample (pH adjusted to 7.2) was mixed with 8 % of PEG 8000 and 0.3 M NaCl. The mixture was incubated at 4°C for about 18 hours. The pellet was collected by centrifugation at $6,700 \times g$ for 30 minutes and dissolved in 10 to 20 ml phosphate buffer saline. The sample was further extracted with equal volume of chloroform and the supernatant was collected by centrifugation at $1,700 \times g$ for 30 minutes. The supernatant was filtered through 0.22 μ M filter and virus particles were further concentrated to approximately 500 μ l by Amicon Ultra-15 centrifugal filter device (Millipore). The sample was loaded to the filter device and centrifuged at $4,000 \times g$ for approximately 10 to 30 min.

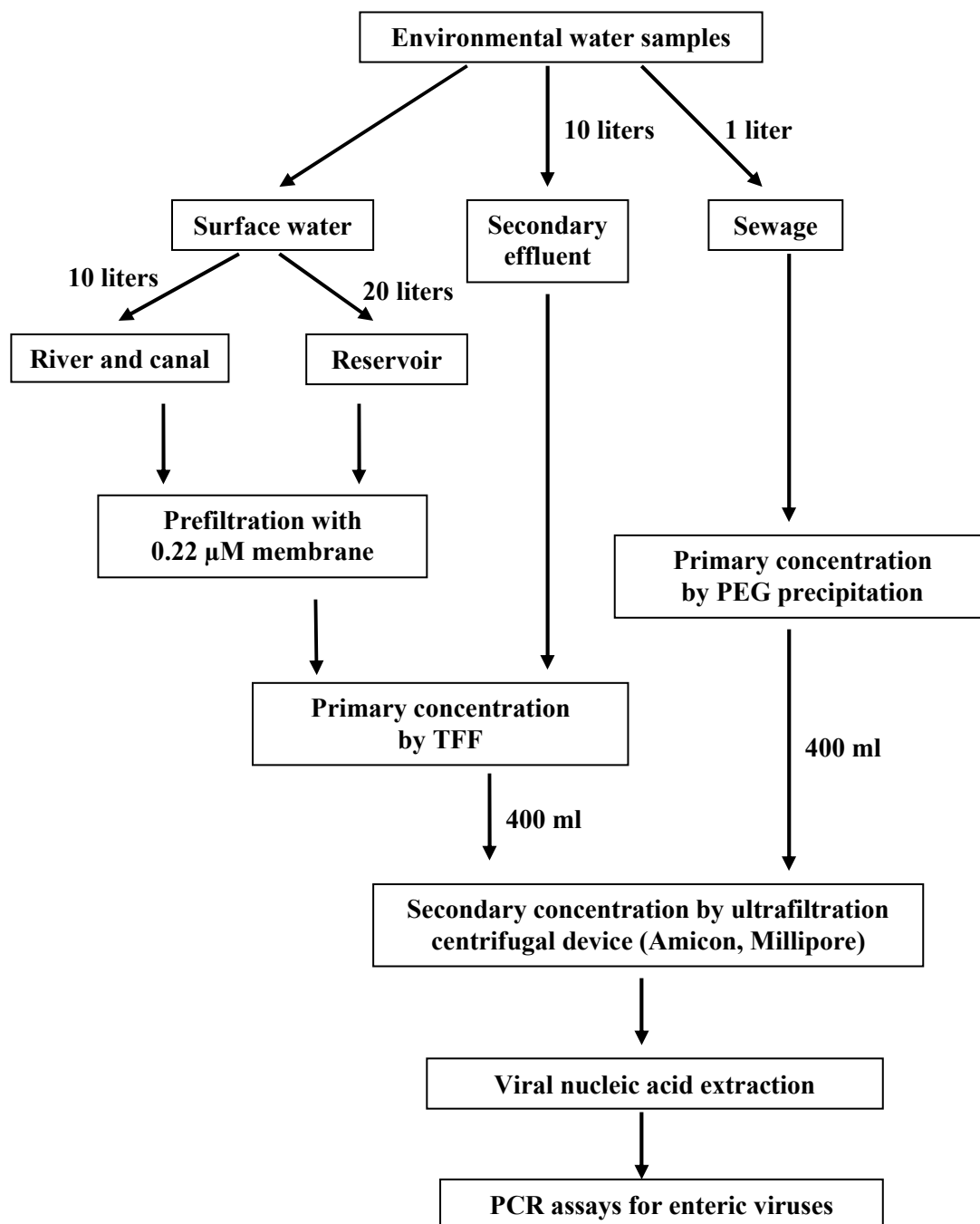


Figure 3.3 Flow chart for major steps in the concentration of enteric viruses from environmental water samples.

3.3.2 Concentration of enteric viruses from water samples by tangential flow filtration

Enteric viruses from surface water samples and secondary effluent were concentrated by using tangential flow filtration (TFF) system with a 30 kDa membrane cassette (Centramate, Pall Corporation). Surface water samples were prefiltered by using an AcroPak 200 capsule filter with a 0.2- μ m-pore-size membrane (Pall Corporation).

The TFF system (Figure 3.4) was assembled as recommended by the manufacturer and connected to a peristaltic pump (model 77410-05; Cole Parmer, Chicago, IL). The system was sanitized with a 0.1 N sodium hydroxide solution and thoroughly flushed with sterile ultrapure water. The water sample was processed until the sample volume remained approximately 200 to 300 ml in the carboy. To enhance the recovery of viruses, elution was performed after the concentration step by the recirculation of sterile glycine (100 ml, 0.05 M, pH 7.0) through the system for 5 min. After completion of each water sample, the system was sanitized and flushed with sterile ultra pure water as recommended by the manufacturer.

A secondary concentration of water samples was done by an Amicon Ultra-15 centrifugal filter device. Briefly, 60 to 120 ml of the TFF-concentrated sample was loaded to the filter device and centrifuged at $4,000 \times g$ for approximately 10 to 30 min. The final volume of each concentrated water sample was approximately 500 μ l.

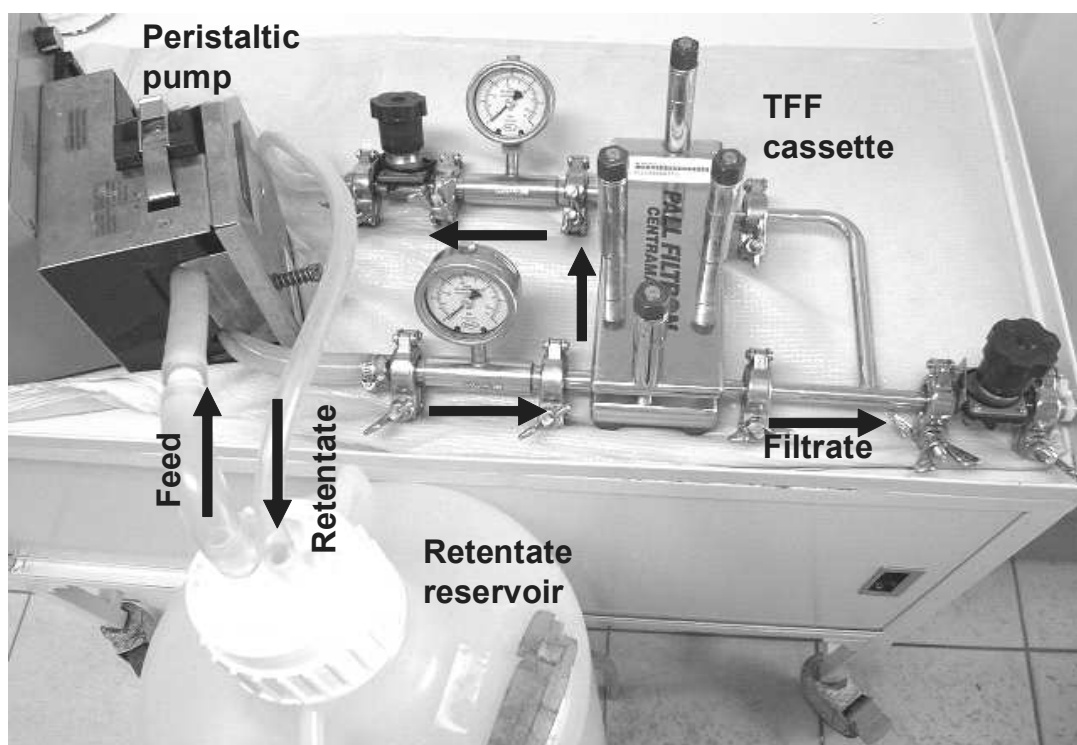


Figure 3.4 Filtration scheme for the small scale tangential flow filtration system with a 30 kDa membrane cassette. During the concentration process, water (feed) is pumped across the membrane surface (TFF cassette). The cross-flow filtration minimizes fouling by sweeping retained molecules off the membrane surface. Filtration is achieved by creating pressure against the membrane in the retentate stream, causing some of the feed solution and dissolved molecules to pass through the membrane as filtrate. Viruses which are larger than the membrane pores are retained in the feed solution and concentrated. The water sample is processed until the volume remained is approximately 200 to 300 ml in the retentate reservoir.

3.4 Detection of bacterial indicators in water samples

Escherichia coli (*E. coli*) and enterococcus fecal bacterial levels in surface water samples were determined by using Colilert 18 and Enterolert (Quantitray/2000) assays (IDEXX Laboratories, Westbrook, Maine), respectively, according to the manufacturer's instructions. Briefly, 10- to 100-fold serial dilutions of each water samples were performed and 100 mL of water samples were subjected to the tests. The most probable number (MPN)/100 mL for both types of bacteria were determined after incubation for 18 hours at 35 °C for Colilert 18 and 24 hours at 41 °C for Enterolert.

3.5 Detection of male-specific RNA coliphage and somatic coliphage by plaque assay

3.5.1 Propagation of coliphages for assay positive controls

Propagation of male-specific RNA coliphage, MS2 (ATCC 15597-B1) and somatic coliphage, phiX174 (ATCC 13706-B1) were conducted using *E. coli* strain, F_{amp} (ATCC 700891), and *E. coli* strain, CN-13 (ATCC 700609), as a host bacterium, respectively (BSI 2001a; BSI 2001b). Briefly, coliphages from a stock solution and log-phase host bacteria were inoculated into 25 mL of tryptic soy (TS) broth and incubated for 4-5 hours. After this, 2.5 mL of chloroform was added to the sample and placed overnight at 4 to 5°C. The aqueous phase of the sample was centrifuged at a minimum of $3,000 \times g$ for 20 min after an overnight incubation. The supernatant containing coliphages was filtered through with 0.2-μm-pore-size membrane filters and stored at 4°C.

3.5.2 Single agar layer for the detection of coliphages

Detection of male-specific RNA coliphages and somatic coliphages in environmental water samples were conducted by using the single agar layer (SAL) procedure (USEPA 2001). A 100 mL surface water sample was assayed by adding

log-phase host bacteria and 100 mL of double-strength molten tryptic soy agar to the sample. Sewage and secondary effluent were 10- to 100-fold diluted and 10 ml of sample was used for plaque assay with 10 ml of double-strength molten tryptic soy agar. Magnesium chloride (0.05 M) was added to the sample to facilitate coliphage adsorption to the added host bacteria. The sample was thoroughly mixed and the total volume was poured into plates. After an overnight incubation (16 to 24 hours), circular lysis zones (plaques) were counted. The quantity of coliphage in a sample was expressed as plaque forming units (PFU) /100 mL. For quality control purposes, a positive control and negative reagent water sample (method blank) was analyzed for each type of coliphage with each sample batch.

3.6 Molecular methods for the detection and characterization of enteric viruses in environmental water samples

3.6.1 Viral nucleic acid extraction

Viral nucleic acid was extracted from 140 µL of the concentrated sample by using a QIAamp Viral RNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Briefly, the sample was first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral nucleic acid. After lysis, the sample was loaded onto the QIAamp silica-gel membrane. Viral nucleic acid was bound to the membrane, and contaminants, which can inhibit downstream enzymatic reactions, were efficiently washed away in two short centrifugation steps using wash buffers. Viral nucleic acid was eluted in 80 µl of DNase-, RNase-free water and stored at -80°C until the PCR assay was performed.

3.6.2 PCR and RT-PCR assays

Oligonucleotide primers used for the PCR amplification of targeted viruses and amplification profiles are listed in Table 3.1. Nested PCR was used for the specific detection of adenoviruses. 5 μ l of extracted nucleic acid was mixed in a total reaction volume of 50 μ l containing 1 $\times$ PCR reaction buffer, 1.5 mM MgCl₂, 200 μ M concentrations of each deoxynucleoside triphosphate, 0.5 μ M concentrations of each forward and reverse primer and 2.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). For nested PCR, the same concentration of reagents and reaction profile were used with 2 μ l of the first PCR product.

Nested RT-PCR was used to detect astroviruses, enteroviruses, noroviruses and hepatitis A viruses in concentrated samples. cDNA was synthesized at 42°C for 60 min in a 10- μ l RT reaction as follow: 5 μ l of template RNA, 1.5 mM MgCl₂, 200 μ M concentrations of each deoxynucleoside triphosphate, 10 units of RNase inhibitor, 0.5 μ l of ImProm-II reverse transcriptase (Promega Corporation, Madison, WI) and 1 μ M concentration of reverse primer for each targeted virus. For the astrovirus detection, an equimolar mixture of reverse primers AstV1a and AstV1b was used.

After reverse transcription, 10 μ l of the cDNA sample was mixed with 1 $\times$ PCR reaction buffer, 1.5 mM MgCl₂ (3.5 mM for enteroviruses detection), 200 μ M concentrations of each deoxynucleoside triphosphate, 0.5 μ M concentration of each forward and reverse primer (0.2 μ M concentration each for noroviruses detection) and 2.5 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems) (1.25 units for noroviruses detection). For nested PCR, the same concentration of reagents and reaction profile were used with 2 μ l of the first PCR product. The amplification products were analyzed on 1.7% agarose gels and visualized under UV illumination after being stained with ethidium bromide.

Table 3.1 Oligonucleotide primers and probes and amplification profile used for nested PCR and RT-PCR assay of enteric viruses

Enteric viruses	Region, amplicon size	Round of PCR	Primer ^a	Sequence (5' – 3') ^b	Amplification profile	Reference
Adenovirus	Hexon gene					
	For detection,	1 st	hexAA1885 (F)	GCCGCAGTGGTCTTACATGCACATC	35 cycles	Puig et al. (1994)
	300-bp	1 st	hexAA1913 (R)	CAGCACGCCCGCGGATGTCAAAGT	(94 °C, 30 sec; 55 °C,	
	143-bp	2 nd	nehexAA1893 (F)	GCCACCGAGACGTACTIONCAGCCTG	30 sec; 72 °C, 1 min)	
		2 nd	nehexAA1905 (R)	TTGTACGAGTACGCCGGTATCCTCGCGGTC		
	For genotyping,	1 st	hex1deg (F)	GCCSCARTGGKWCWTACATGCACATC	35 cycles	Allard et al. (2001)
	301-bp	1 st	hex2deg (R)	CAGCACSCCICGRATGTCAAA	(94 °C, 30 sec; 55 °C,	
Astrovirus	ORF 2	2 nd	nehex3deg (F)	GCCCCGYGCMACIGAIACSTACTTC	30 sec; 72 °C, 1 min)	
		2 nd	nehex4deg (R)	CCYACRGCCAGIGTRWAICGMRCYTTGTA		
	449-bp	1 st	AstV1a (R)	TCAGATGCATTRTCRTTIGT	35 cycles	Oh et al. (2003)
		1 st	AstV1b (R)	TCTGAAGCATTRTCRTTIGT	(94 °C, 30 sec; 42 °C,	
		1 st	Mon269 (F)	CAACTCAGGAAACACAGGGTGT	30 sec; 72 °C, 45 sec)	
	412-bp	2 nd	Mon244 (F)	GGTGTCACAGGACCAAAACC		
		2 nd	AstV3 (R)	TTTGTIARCCACCAICCRTC		

Table 3.1 (Continued)

Enteric viruses	Region, amplicons size	Round of PCR	Primer^a	Sequence (5' – 3')^b	Amplification profile	Reference
Enterovirus	5' NTR				35 cycles	Hafliger et al. (1997)
	438-bp	1 st and 2 nd	EV6 (F)	CAAGCACTTCTGTTCCTCCCGG	(94 °C, 30 sec; 60 °C, 20 sec; 72 °C, 1 min)	
	400-bp	1 st	EV3 (R)	ATTGTCACCATAAAGCAGCCA		
		2 nd	EV5 (R)	CACGGACACCCCAAAGTA		
Hepatitis A virus	5' NTR (for detection)					Pina et al. (2001)
	368-bp	1 st	HAV1 (F)	TTGGAACGTCACCTTGCA GTG	35 cycles	
		1 st	HAV2 (R)	CTGAGTACCTCAGAGGCAAAAC	(95 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min)	
	290-bp	2 nd	neHAV1 (F)	ATCTCTTTGATCTTCCACAAG		
		2 nd	neHAV2 (R)	GAACAGTCCAGCTGTCAATGG		
	VP1/2A (for genotyping)					
	532-bp	1 st	HHA1 (F)	TGCAAAATTAYAAAYCAYTCTGATGA	35 cycles	
		1 st	HHA2 (R)	TTTCTGTCCATTITYTCATCATTC	(95 °C, 1 min; 42 °C, 1 min; 72 °C, 1 min)	
	436-bp	2 nd	HHA3 (F)	TTYAGTTGYTAYTTGTCTGT		
		2 nd	HHA4 (R)	TCAAGAGTCCACACACTTC		

Table 3.1 (Continued)

Enteric viruses	Region, amplicon size	Round of PCR	Primer ^a	Sequence (5' – 3') ^b	Amplification profile	Reference
Norovirus	Capsid gene				40 cycles	Kojima et al.
GI	381-bp	1 st	COG1F (F)	CGYTGATGCGNTTYCATGA	(94 °C, 1 min; 50 °C, 1 min; 72 °C, 2 min)	(2002); Kageyama et al. (2003)
		1 st and 2 nd	G1SKR (R)	CCAAACCCARCCATTCTACA		
	330-bp	2 nd	G1SKF (F)	CTGCCCCGAATTYGTAAATGA		
GI	399-bp	1 st	COG2F (F)	CARGARBCNATGTTYAGRTGGATGAG		
		1 st and 2 nd	G2SKR (R)	CCRCCNGCATRHCCRTTCTACAT		
	344-bp	2 nd	G2SKF (F)	CNTGGGAGGGCGATCGCAA		

^a F = Forward primer, R = Reverse primer

^b Mixed bases in degenerated primers: S = C/T, R = A/G, K = G/T, D = G/A/T, W = A/T, Y = C/T, M = A/C, H = A/T/C, B = C/G/T, N = A/C/G/T. Note that inosine (I) has base-pairing bias for C, A, G, and then T.

Standard precautions were applied when conducting the PCR assay, such as UV sterilization of PCR equipment and the working environment, using aerosol-resistant tips, separate locations for sample preparation and amplification. Negative controls using PCR-grade water and positive controls were included in each reaction set. For noroviruses, diluted sewage samples (positive with noroviruses by RT-PCR and DNA sequencing) were included as positive controls.

3.6.3 Molecular cloning and DNA sequencing

The nested PCR products were purified by using the Wizard SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI) prior to sequencing. The PCR products were sequenced from both directions with the ABI Big Dye Terminator cycle sequencing Kit and Prism 3100 genetic analyzer.

It was difficult to sequence directly the PCR products contained noroviruses and enteroviruses because of the presence of multiple virus strains. Thus PCR products were cloned into the pGEM-T Vector System (Promega Corporation, Madison, WI) and then at least three colonies were sequenced. To make a 10 µl of ligation reaction, 1 µl ultrapure water, 5 µl ligation buffer, 1 µl pGEM-T vector, 2 µl purified PCR product and 1 µl T4 DNA ligase were mixed. The reaction was incubated overnight at 4°C. JM109 competent *E. coli* cells were used for the transformation of ligated DNA. 2 µl of each ligation reaction were transferred into cells and the tube was flicked gently to mix. The mixture was placed on ice for 20 min. The cells were heat shocked for 45-50 seconds in a water bath at exactly 42 °C and then placed on ice. 950 µl of SOC medium were added to each tube containing cells transformed with the ligation reaction and incubated for 1.5 hours at 37 °C with shaking at 150 rpm. 120 µl of each transformation culture was plated onto duplicate LB agar containing 100 µg/ml ampicillin, 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal. The plates were incubated overnight (16 to 24 hours) at 37 °C.

At least three white colonies were randomly selected and grew overnight at 37 °C in 5 ml LB broth containing 100 µg/ml ampicillin. Plasmid DNA was isolated from the colonies by using QIAprep Spin Miniprep Kit (Qiagen, Germany) according to the manufacturer's instructions.

3.6.4 Quantitative real-time PCR and RT-PCR (QPCR)

Real-time PCR is a relatively new molecular biology technique to quantitatively detect enteric viruses in various environmental water samples. This technique enables high sample throughput in very short analysis time (less than one hour) and offers excellent specificity.

The LightCycler 2.0 Instrument and LightCycler kits for real-time PCR (Roche Diagnostics GmbH, Mannheim, Germany), coupled with TaqMan probe, were used in this study. The LightCycler 2.0 Instrument monitors amplification of a PCR product simultaneously, in real-time and online, with six different fluorescence detection channels (530, 560, 610, 640, 670 and 705 nm). The dual-labeled fluorogenic probe or TaqMan probe is a single stranded oligonucleotide labelled with two different dyes, a reporter fluorescent dye at the 5' end and a quencher dye at the 3' end. During the elongation step of real-time PCR, the *Taq* DNA polymerase 5' exonuclease activity hydrolyses the probe bound to the specific DNA template. This cleavage releases the reporter dye from the probe and away from the quencher dye, resulting in fluorescence that is detected by the instrument.

3.6.4.1 Quantitative real-time PCR and RT-PCR protocols

Oligonucleotide primers and probes used for the QPCR amplification of adenoviruses, noroviruses GI and GII and astroviruses are listed in Table 3.2.

To construct quantitative standards for adenoviruses, a section of adenovirus type 40 hexon gene was PCR amplified with a primer set specific for adenovirus type 40 (forward primer: 5'-TGGCCACCCCCTCGATGA-3', reverse primer: 5'-TTTGGGGGCCAGGGAGTTGTA-3') (Jothikumar et al., 2005a). For norovirus GI, norovirus GII and astrovirus type 1, primer pair COG1F/COG1R, JJV2F/COG2R and AV1/AV2 (Table 3.2), respectively, was used. The PCR product was subsequently cloned into pGEM-T Vector System (Promega Corporation, Madison, WI) as described in the section 3.6.3. Plasmid DNA was linearized and purified by using the Wizard SV Gel and PCR Clean-up System (Promega Corporation, Madison, WI). The concentration of purified plasmid DNA was determined by Nanodrop ND 1000 spectrophotometer (Nanodrop, Wilmington, DE). The plasmid DNA was 10-fold serially diluted in nuclease-free water and standard curves were generated using 10 to 10^5 copies of plasmid DNA.

Amplification reaction mixtures for adenoviruses (total final volume of 20 μ l in glass capillaries) contained 5 μ l template DNA or standard, 4 μ l of 5 $\times$ LightCycler TaqMan Master Mix, 250 nM of each primer and 150 nM of probe. The thermal cycling protocols for adenovirus QPCR were as follows: 15 min at 95°C for initial denaturation, followed by 45 cycles of three steps consisting of 10 s at 95°C, 30 s at 55°C, and 15 s at 72°C (at a temperature transition rate of 20°C/s), with a cooling step at 40°C for 30 s.

Table 3.2 Oligonucleotide primers and probes used for QPCR assay of enteric viruses

Enteric viruses	Region	Primer ^a and probe	Sequence (5' – 3') ^b	Reference
Adenovirus	Hexon gene	JTVXF (F)	GGACGCCCTCGGAGTACCTGAG	Jothikumar et al. (2005a)
		JTVXR (R)	ACIGTGGGGTTTCTGAACTTGTT	
		JTVXP	FAM-CTGGTGCAGTTCGCCCCGTGCCA-BHQ1	
Norovirus	Capsid gene			Kageyama et al. (2003);
GI		COG1F (F)	CGYTGGATGCGNTTYCATGA	Jothikumar et al. (2005b)
		COG1R (R)	CTTAGACGCCATCATCATTYAC	
		JJV1P	FAM-TGTGGACAGGAGATCGCAATCTC-BHQ1	
		JJV2F (F)	CAAGAGTCAATGTTTAGGTGGATGAG	
		COG2R (R)	TCGACGCCATCTTCATTCACA	
GII		RING2-TP	FAM-TGGAGGGCGATCGCAATCT-BHQ1	
Astrovirus	3'-end consensus	AV1 (F)	CCGAGTAGGATCGAGGGT	Le Cann et al. (2004)
	region	AV2 (R)	GCTTCTGATTAAATCAATTTTAA	
		AVs	FAM-CTTTTCTGCTCTGTTAGATTATTTAATCACC-BHQ1	

^a F = Forward Primer, R = Reverse primer^b Mixed bases in degenerated primers: Y = C/T, N = A/C/G/T. Note that inosine (I) has base-pairing bias for C, A, G, and then T.

For the detection of noroviruses and astroviruses, cDNA was synthesized at 42°C for 60 min in a 10- μ l RT reaction as follow: 5 μ l of template RNA, 1.5 mM MgCl₂, 200 μ M concentrations of each deoxynucleoside triphosphate, 10 units of RNase inhibitor, 0.5 μ l of ImProm-II reverse transcriptase (Promega Corporation, Madison, WI) and 1 μ M concentration of reverse primer for each targeted virus. Amplification reaction mixtures (total final volume of 20 μ l in glass capillaries) contained 5 μ l cDNA, 4 μ l of 5 $\times$ LightCycler TaqMan Master Mix, 500 nM of each primer and 100 nM of probe. The thermal cycling protocols for both norovirus and astrovirus QPCR were as follows: 15 min at 95°C for initial denaturation, followed by 45 cycles of three steps consisting of 10 s at 95°C, 30 s at 55°C, and 15 s (1 s for norovirus GI and astrovirus detection) at 72°C, (at a temperature transition rate of 20°C/s), with a cooling step at 40°C for 30 s.

The environmental samples and standards were each run at least in triplicate. A negative control (PCR grade water without template) and positive control were included in all PCR runs. The crossing point (CP) of each PCR assay was automatically determined by the LightCycler program, version 4.0 and was used to calculate the viral gene concentrations

3.7 Clinical samples for noroviruses

Stool samples and rectal swabs from several gastroenteritis outbreaks in Singapore between August 2006 and January 2007 were collected and analyzed at the Molecular Laboratory, Department of Pathology, Singapore General Hospital. In addition, stool samples from sporadic gastroenteritis cases during this period were also included in the study.

3.7.1 RNA extraction, PCR amplification, and sequencing for noroviruses in clinical samples

Viral RNA from stool samples and rectal swabs was extracted by using the QIAamp Stool Mini Kit (Qiagen, Germany) and QIAamp viral RNA Mini Kit (Qiagen, Germany), respectively, according to the manufacturer's instructions. Rectal swab samples were resuspended in 200 to 400 μ L of phosphate-buffered saline prior to the RNA extraction.

Extracted RNA was first tested by real-time RT-PCR for noroviruses and to determine genogroup (GI or GII). This was performed on the Roche Lightcycler 1.2 (Roche Diagnostics) using the protocol described by Kageyama et al. (2003) with modifications. These primers target the open reading frame 1 (ORF1)-ORF2 junction. For GI PCR, 4 μ L of extracted RNA was added to 6 μ L of PCR amplification mix, comprising 3.75 μ L of Roche Lightcycler RNA Master HybProbe (Roche Diagnostics), 0.75 μ L of manganese acetate, 4 pmoles each of primers COG1F and COG1R (Table 3.2), 2 pmoles each of probes RING1(a)-TP (FAM-AGATY GCGATCYCCTGTCCA-TAMRA) and RING1(b)-TP (FAM-AGATCGCGGTCT CCTGTCCA-TAMRA) and 0.3 μ L of nuclease free water. For GII PCR, 4 μ L of extracted RNA was added to 6 μ L of PCR amplification mix, comprising 3.75 μ L of Roche Lightcycler RNA Master HybProbe, 0.75 μ L of manganese acetate, 3 pmoles each of primers COG2F (5'-CARGARBCNATGTTYAGRTGGATGAG-3') and COG2R (Table 3.2), 1.75

pmoles of probe RING2-TP (Table 3.2) and 0.575 μ L of nuclease free water. Positive and negative controls were used for each run. In addition, for each sample, an inhibition control was set up by adding 4 μ L of the RNA extract, 1 μ L of norovirus GI- or GII-positive control and 6 μ L of the amplification mix. The thermal cycling protocols for both GI and GII PCR were as follows: 20 min at 61°C for reverse-transcription, then 2 min at 95°C for initial denaturation, followed by 60 cycles of three steps consisting of 5 s at 95°C, 12 s at 56°C, and 20 s at 72°C, with a cooling step at 40°C for 30 s.

Extracted RNA that was positive for norovirus was subsequently amplified as previously described (Kageyama et al., 2004), targeting the capsid gene. This yielded amplicons of approximately 468 bp in size which were then sequenced using the ABI Avant 3100 (Applied Biosystems).

3.8 Phylogenetic analysis

Nucleotide sequences were edited and aligned with the BioEdit sequence alignment editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). The sequences were compared to other reference sequences available in the GenBank database. Phylogenetic distances were determined by the Kimura's two parameter method using the DNADIST program of the PHYLIP package, version 3.67 (<http://evolution.genetics.washington.edu/phylip.html>). A phylogenetic tree was generated by the NEIGHBOR program. The robustness of the grouping was determined by bootstrap resampling of the multiple sequence alignments (100 sets) with the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE. The output graphics of the trees were created with the TREEVIEW package (Page 1996). Bootstrap values of 70 or higher were considered statistically significant for the grouping.

3.9 Statistical analyses

The statistical analyses were performed with the Statistical Package for Social Sciences for Windows (SPSS Inc.) software. Rainfall data of the closest rainfall gauge stations for Marina catchments were obtained from the National Environment Agency (NEA), Singapore. An independent-samples t-test was conducted to compare the rainfall intensity for dry (June to August, October 2006) and wet weather (November to December 2006) seasons. The same analysis was used to determine possible significant differences for the levels of *E. coli* and enterococcus for dry and wet weather seasons. A chi-square analysis was used to determine if the detection of enteric viruses was significantly more likely during the dry versus the wet season. A binary logistic regression model was used to analyze the relationship between the occurrence of enteric viruses and levels of bacterial indicators. Each virus was treated as a binary variable; that is, a score of 0 was assigned when the virus was not detected, and a score of 1 was assigned when the virus was detected. A Spearman's rho rank order correlation was used to determine correlations in the quantitative detection between coliphages and enteric viruses in sewage. In all cases, significance was determined at the 95% confidence level.

Chapter 4

Occurrence and Prevalence of Enteric Viruses in Tropical Urban Surface Waters and Wastewaters

4.1 Introduction

The increasing scarcity of natural fresh water resources, particularly in highly urbanized areas, makes the harvesting of urban stormwater a valuable water resource. Urban reservoirs in Singapore, besides being important sources of drinking water supplies, have been used for recreational purposes particularly for various water sports. Therefore, there is a great need for studying microbiological water quality of highly urbanized water catchment areas in order to maintain the quality and safety of water systems used for drinking water supply and recreation.

Sewage has been recognized as an important source of waterborne pathogens in urban watersheds. It has been suggested that unprecedented and rapid increases in population and the subsequent increase in sewage discharges contribute to surface water quality impairment worldwide (Rose and Molloy 2007). Human fecal matter in sewage contains a diverse group of viruses that causes gastrointestinal infections, hepatitis or neurological diseases. These viruses, collectively known as enteric viruses are shed in high concentrations in fecal matter of infected patients and are discharged into sewage which may contaminate surface water sources for drinking water, recreational activities, aquaculture and irrigation. Enteric viruses are transmitted primarily via the fecal-oral route and pose a significant public health threat in water environments (Metcalf et al., 1995). A growing number of pathogenic human viruses can readily be detected in untreated and treated wastewaters (Sedmak et al., 2005; van den Berg et al., 2005; Meleg et al., 2006; Katayama et al., 2008; Symonds et al., 2009).

Fecal indicator bacteria such as fecal coliforms and *Escherichia coli* have been used by water quality regulators worldwide for over a century as standard tools to monitor human fecal contamination in water. An increased knowledge of the diversity and sources of pathogenic viruses and their persistence in water environments raises critical questions about the suitability of the current bacterial indicator approaches for assessing the human waste contamination of waters (Savichtcheva and Okabe 2006). Moreover, media-based assays for the enumeration of bacterial indicators in water cannot discriminate the source of fecal contamination between human and animal (Griffin et al., 2001). Recent advances in molecular techniques make it timely to reassess the current paradigm of relying predominantly on traditional bacterial indicators for waterborne pathogenic viruses (Robertson and Nicholson 2005). Given that direct pathogen detection techniques are now available, direct surveillance for pathogenic viruses may be warranted to better protect public health (Fong and Lipp 2005). Several studies have suggested that adenoviruses are present in the greatest numbers compared to other viruses in wastewater and are thus, a potential index for viral pollution in water environments (Pina et al., 1998; Albinana-Gimenez et al., 2006; Symonds et al., 2009). However, it is necessary to develop a baseline understanding of diverse groups of enteric viruses occurring in wastewater in specific regions in order to determine which viruses are effective indicators of fecal contamination (Symonds et al., 2009). Currently no baseline data on the occurrence of human enteric viruses in raw sewage and environmental waters has been reported in Singapore.

Enteric viruses in the urban water environment may be transported by stormwater runoff, combined and sanitary sewer overflows, and discharge of wastewater treatment plant effluent. The significance of stormwater runoff in urban catchments and waterways has received increased attention in recent years. Previous studies have demonstrated that urban runoff contributes to the virological contamination of receiving waters and the resulting impairment of recreational water quality (Grohmann et al., 1993; Jiang et al., 2001; Jiang and Chu 2004).

This chapter describes the virological investigation of surface waters from Marina Bay and its catchment areas. Marina Bay has been converted to the most highly urbanized freshwater reservoir in Singapore and serves as an important water source for various recreational activities and future drinking water supply. In addition, the occurrence of human enteric viruses in urban wastewaters of Singapore was studied. The overall goal of the present study was to gain insight into the prevalence and abundance of viral pathogens in tropical aquatic environments by using molecular methods. Knowledge on the presence of human enteric viruses in water environments contributes to the understanding of the mechanisms of viral transmission and the possible role played by water as a vehicle of transmission.

4.2 Results

4.2.1 Occurrence of enteric viruses in urban wastewaters from water reclamation plants

Sewage and secondary effluent samples in water reclamation plants (Bedok, Kranji and Ulu Pandan) were collected and analyzed monthly from January to June 2007. Coliphages and all five tested human enteric viruses (adenoviruses, astroviruses, enteroviruses, noroviruses and hepatitis A viruses) were detected in wastewater samples collected from all water reclamation plants. Somatic coliphages, male-specific coliphages, adenoviruses, astroviruses and both noroviruses GI and GII were found in 100% of the sewage and secondary effluent samples. Enteroviruses were detected in 94% of sewage and 89% secondary effluent, and hepatitis A viruses were detected in 78% of sewage and 28% of secondary effluent. Examples of RT-PCR products visualized by gel electrophoresis for the detection of hepatitis A viruses in raw sewage and astroviruses in secondary effluent are shown in Figure 4.1 and Figure 4.2, respectively.

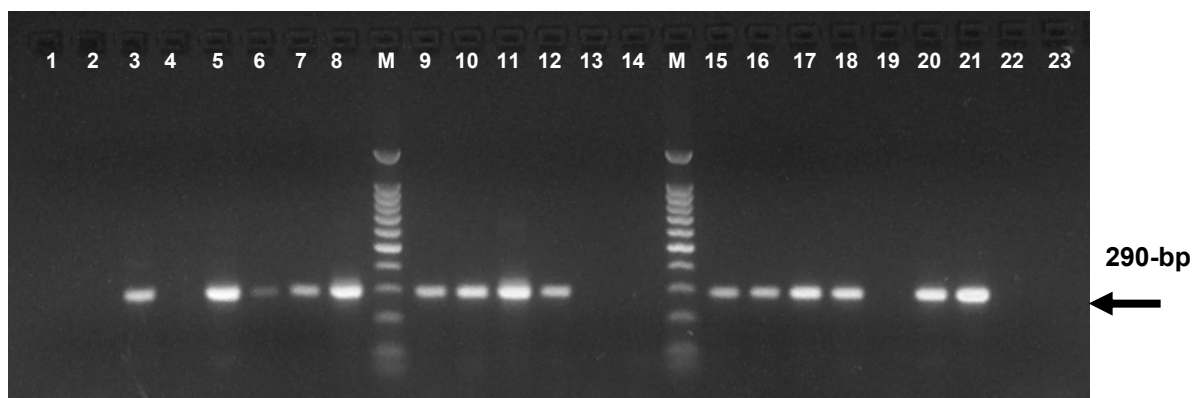


Figure 4.1 Gel photograph of hepatitis A viruses detected in urban sewage samples. Lanes: M, 100-bp DNA marker; 1 and 2, RT-PCR negative controls; 3 to 20, sewage positive/negative for hepatitis A viruses (290-bp amplicons); 21, positive control; 22 and 23, RT-PCR negative controls. Hepatitis A viruses were not detected in sewage samples collected in January, April and June 2007 from Kranji Water Reclamation Plant (WRP) and in April 2007 from Ulu Pandan WRP.

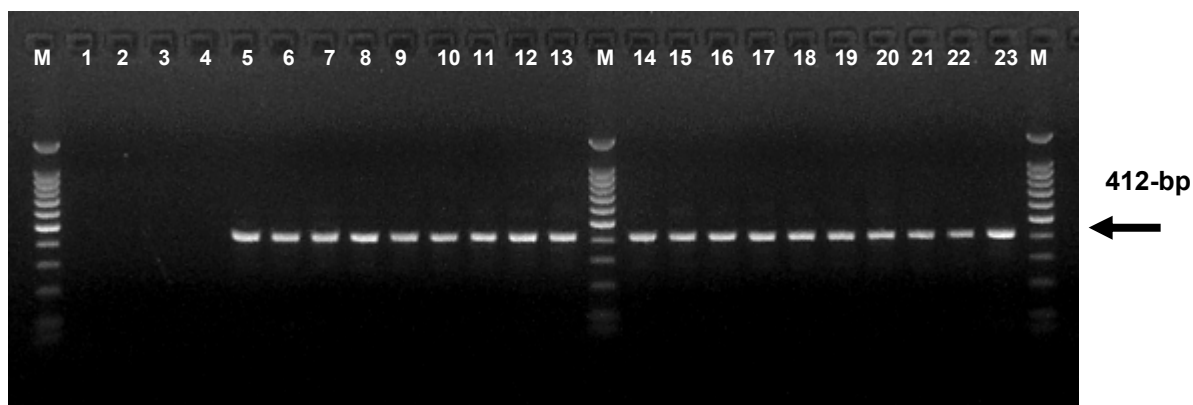


Figure 4.2 Gel photograph of human astroviruses detected in secondary effluent. Lanes: M, 100-bp DNA marker; 1 to 4, RT-PCR negative controls; 5 to 22, secondary effluent positive for astroviruses (412-bp amplicons); 23, positive control.

4.2.2 Quantitative detection of viruses in wastewater samples

Quantitative data of enteric viruses in environmental samples are important for assessing microbial risk in water. In the present study, concentration of human enteric viruses in sewage and secondary effluent was estimated by quantitative real-time PCR. Somatic and male-specific coliphages in sewage and secondary effluent were quantified by plaque assays.

In raw sewage, the concentrations of somatic coliphages (average, 1.8×10^5 PFU/100ml) were higher than the concentrations of male-specific coliphages (average, 4.3×10^4 PFU/100ml). On average, somatic and male-specific coliphages were present at 10^2 PFU/100ml in secondary effluent. Virus removal by biological treatment processes was on average 2.4 log₁₀ units for both coliphages (Table 4.1).

The real-time PCR protocols for astroviruses, adenoviruses and noroviruses GI and GII were optimised for their application in the LightCycler real-time PCR platform. The amplification conditions tested included variations of oligonucleotide concentration for each primer from 0.2 μ M to 1.0 μ M, and variations of probe concentration from 0.05 μ M to 0.2 μ M. The optimal primer and probe concentrations (in Materials and Methods) were selected based on their high sensitivity (low crossing point, CP value) and reaction efficiency (> 90%). The standard curves used for quantifying enteric viruses in concentrated samples are shown in Figure 4.3. All assays were linear over five orders of magnitude. The QPCR detection limit was approximately 6 gene copies per reaction for adenoviruses and 10 gene copies per reaction for astroviruses, noroviruses GI and noroviruses GII.

Table 4.1 The range and average concentration of somatic and male-specific coliphages in urban wastewaters collected from water reclamation plants in Singapore

Sampling location	Wastewater type ^a	Average concentration (min - max)	
		Somatic coliphages (PFU/100ml)	Male-specific phages (PFU/100ml)
Bedok	Sewage	2.2×10^5 ($4.0 \times 10^4 - 3.8 \times 10^5$)	5.4×10^4 ($3.6 \times 10^4 - 7.3 \times 10^4$)
	Secondary effluent	7.0×10^2 ($5.0 \times 10^2 - 1.1 \times 10^3$)	1.6×10^2 ($90 - 2.6 \times 10^2$)
	Calculated log removal	2.5	2.5
Kranji	Sewage	1.1×10^5 ($3.5 \times 10^4 - 1.9 \times 10^5$)	4.4×10^4 ($3.0 \times 10^3 - 1.1 \times 10^5$)
	Secondary effluent	6.9×10^2 ($3.2 \times 10^2 - 1.3 \times 10^3$)	1.7×10^2 ($40 - 6.0 \times 10^2$)
	Calculated log removal	2.2	2.4
Ulu Pandan	Sewage	2.0×10^5 ($1.3 \times 10^5 - 4.4 \times 10^5$)	3.1×10^4 ($1.6 \times 10^4 - 6.0 \times 10^4$)
	Secondary effluent	5.8×10^2 ($3.2 \times 10^2 - 9.5 \times 10^2$)	1.4×10^2 ($50 - 2.6 \times 10^2$)
	Calculated log removal	2.5	2.4

^a Sewage and secondary effluent samples were collected monthly from each of the three water reclamation plants from January to June 2007. A total of 18 sewage and 18 secondary effluent samples were analyzed for coliphages by plaque assay.

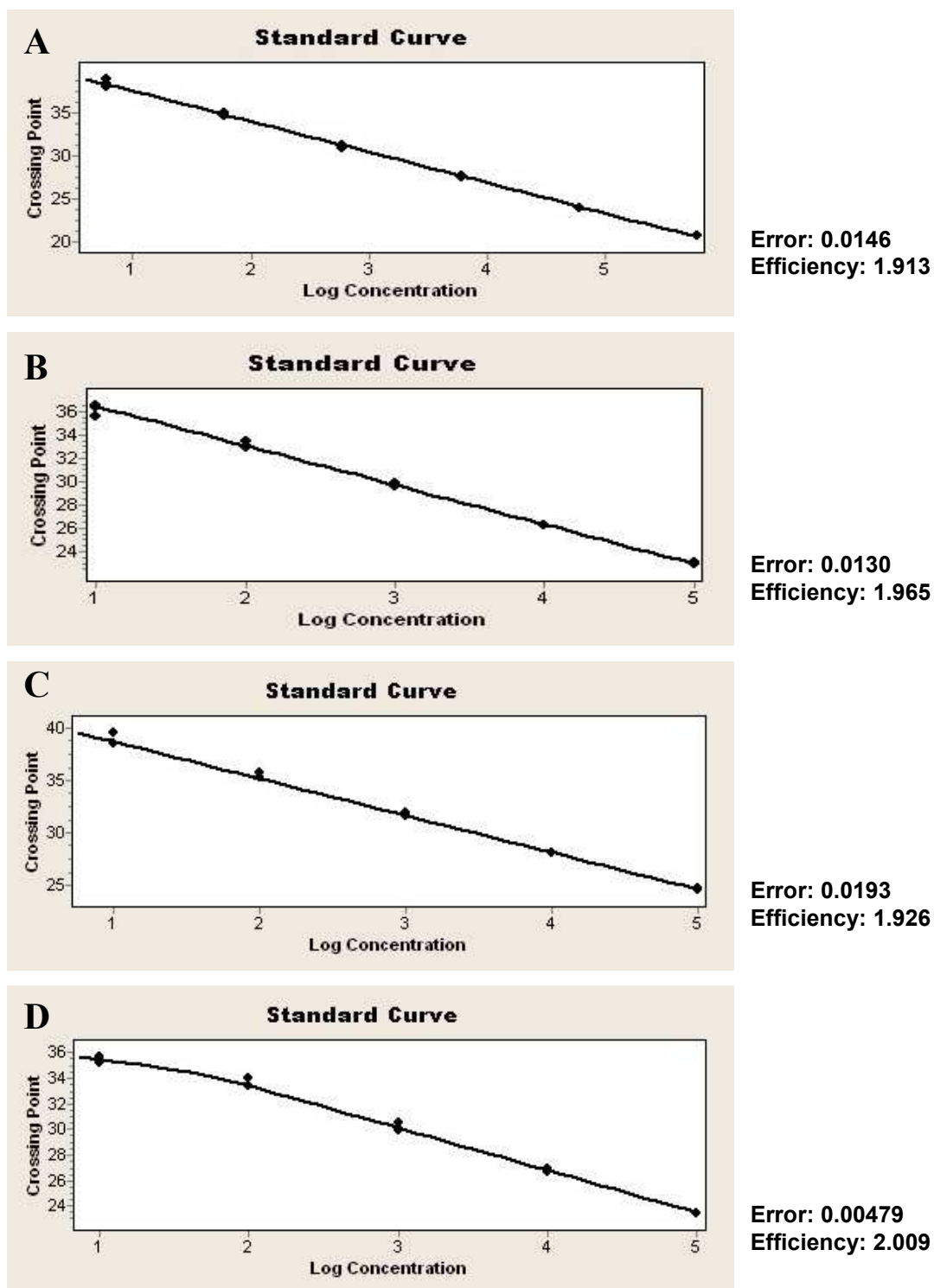


Figure 4.3 QPCR standard curves for (A) adenoviruses, (B) astroviruses, (C) noroviruses GI and (D) noroviruses GII.

A comparison of concentrations of somatic coliphages, male-specific coliphages, adenoviruses, noroviruses GI and GII and astroviruses in sewage and secondary effluent are shown in Figure 4.4 in a box plot format. Astrovirus genome concentrations in the sewage (average of 3.7×10^6 copies/100ml) were 1 to 2 orders of magnitude higher than adenovirus, norovirus GI and norovirus GII genome concentrations (9.4×10^4 , 3.2×10^5 and 2.3×10^5 copies/100ml, respectively) during the study period. Viral genome concentrations were less variable in secondary effluent samples, with average of 1.0×10^3 , 3.9×10^3 , 7.1×10^3 and 5.2×10^3 copies/100ml for adenovirus, astrovirus, norovirus GI and norovirus GII, respectively. For adenoviruses and astroviruses, the average removal by biological treatment processes was 2.0 and 3.0 $\log_{10}$ units, respectively. For noroviruses GI and GII, removal was relatively lower, with 1.7 and 1.6 $\log_{10}$ units on average, respectively (Table 4.2). It should be noted that the final viral concentration quantified by QPCR was not corrected by the probable loss during viral concentration and reverse transcription steps. Therefore, the figures presented here are estimated levels of enteric viruses in the environment.

Statistical analysis by the Spearman's rho showed significant correlations in quantitative detection between somatic coliphages and adenoviruses ($P < 0.05$) and between male-specific coliphages and noroviruses GII ($P < 0.01$) in sewage samples.

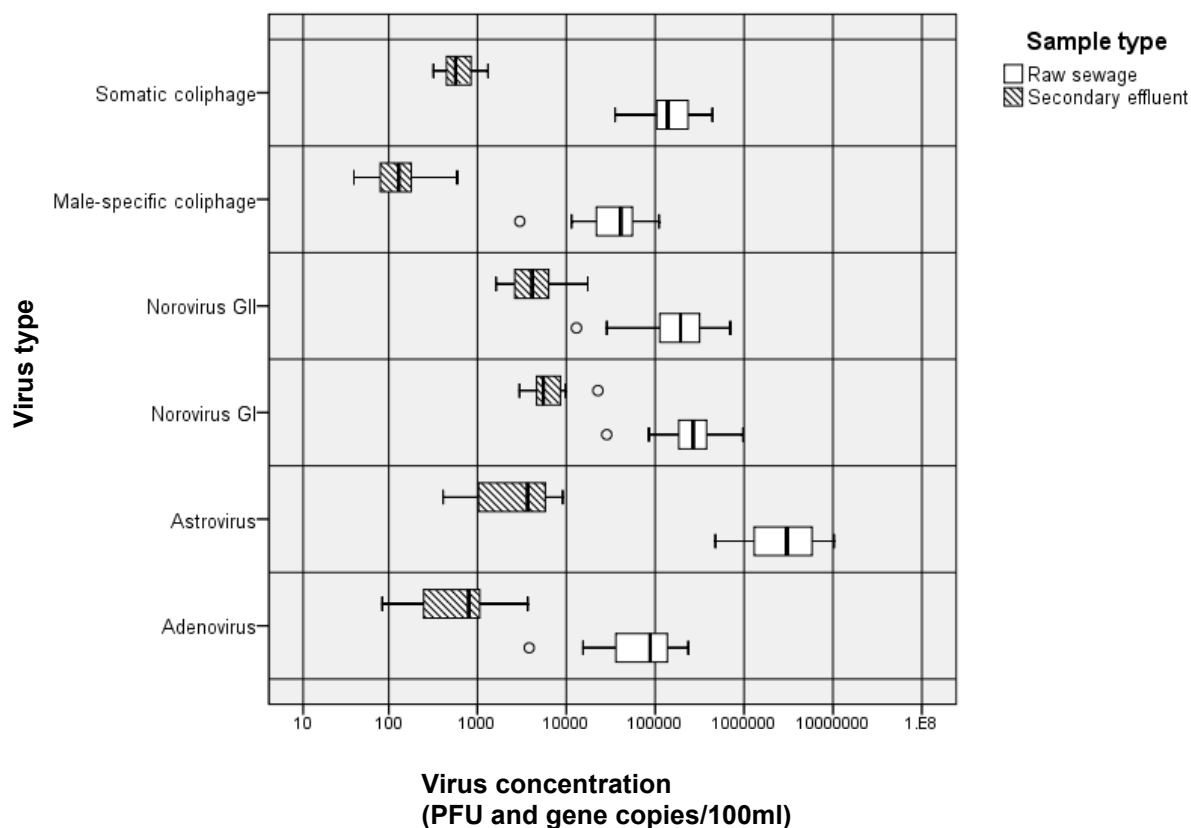


Figure 4.4 Boxplot comparison of the concentrations of somatic and male-specific coliphages (PFU/100ml) and enteric viruses (gene copy number/100ml) isolated from raw sewage (n = 18) and secondary effluent (n = 18). The box represents 50% of the data values. The line across the inside of the box represents the median value and the lines extending from the box represent the 95% confidence intervals. Outliers are represented by circles.

Table 4.2 The range and average concentration of adenoviruses, noroviruses GI and GII and astroviruses in urban wastewaters collected from water reclamation plants in Singapore

Sampling location	Wastewater type	Average concentration (min - max)			
		Adenoviruses (gene copies/100ml)	Noroviruses GI (gene copies/100ml)	Noroviruses GII (gene copies/100ml)	Astroviruses (gene copies/100ml)
Bedok	Sewage (n = 6)	1.3×10^5 ($2.7 \times 10^4 - 2.4 \times 10^5$)	3.7×10^5 ($8.5 \times 10^4 - 9.7 \times 10^5$)	3.5×10^5 ($3.3 \times 10^4 - 7.0 \times 10^5$)	4.9×10^6 ($1.1 \times 10^6 - 1.0 \times 10^7$)
		6.9×10^2 ($1.2 \times 10^2 - 1.6 \times 10^3$)	9.0×10^3 ($4.7 \times 10^3 - 2.3 \times 10^4$)	6.7×10^3 ($1.6 \times 10^3 - 1.8 \times 10^4$)	4.1×10^3 ($4.6 \times 10^2 - 7.1 \times 10^3$)
	Calculated log removal	2.3	1.6	1.7	3.1
Kranji	Sewage (n = 6)	4.3×10^4 ($3.8 \times 10^3 - 1.1 \times 10^5$)	2.7×10^5 ($2.9 \times 10^4 - 6.1 \times 10^5$)	1.3×10^5 ($1.3 \times 10^4 - 2.4 \times 10^5$)	3.2×10^6 ($4.8 \times 10^5 - 8.5 \times 10^6$)
		1.4×10^3 ($1.4 \times 10^2 - 3.0 \times 10^3$)	4.6×10^3 ($3.0 \times 10^3 - 6.1 \times 10^3$)	3.7×10^3 ($1.9 \times 10^3 - 5.1 \times 10^3$)	3.7×10^3 ($4.3 \times 10^2 - 8.9 \times 10^3$)
	Calculated log removal	1.5	1.8	1.5	3.0
Ulu Pandan	Sewage (n = 6)	1.1×10^5 ($4.3 \times 10^4 - 1.8 \times 10^5$)	3.1×10^5 ($9.9 \times 10^4 - 5.2 \times 10^5$)	2.1×10^5 ($6.4 \times 10^4 - 4.9 \times 10^5$)	2.9×10^6 ($1.3 \times 10^6 - 6.7 \times 10^6$)
		9.8×10^2 ($84 - 3.7 \times 10^3$)	7.2×10^3 ($4.0 \times 10^3 - 9.6 \times 10^3$)	5.0×10^3 ($2.0 \times 10^3 - 1.0 \times 10^4$)	3.8×10^3 ($4.1 \times 10^2 - 9.1 \times 10^3$)
	Calculated log removal	2.1	1.6	1.6	2.9

4.2.3 Detection of microbial water quality indicators and enteric viruses in urban surface waters

In the present study, 78 water samples were collected from catchment areas of Marina Bay from June 2006 to January 2009. Six water samples of the bay which is fed by the discharges from the catchment waterways were also collected from March to July 2007.

Over the course of the study, *E. coli* levels ranged between a most probable number (MPN) of 180 per 100 ml and an MPN number of 8.2×10^5 per 100 ml (Table 4.3). Enterococcus levels in surface water samples ranged between 10 and 19,680 MPN per 100 ml.

Enteric viruses in environmental water samples were concentrated by tangential flow filtration. The recovery rate of viruses by tangential flow filtration was determined using catchment water samples seeded with male-specific RNA bacteriophages, MS2 (ATCC 15597-B1). These phages are a group of single-stranded RNA viruses and have physical properties comparable to the noroviruses. The average recovery of bacteriophages MS2 was 56 to 70% as determined by plaque assay.

Table 4.3 The range and geometric mean MPN *Escherichia coli* and enterococcus counts at each sampling station

Sampling site	Number of sample tested	<i>Escherichia coli</i> (MPN/100ml)			Enterococcus (MPN/100ml)		
		Geometric mean	Range (min - max)		Geometric mean	Range (min - max)	
Singapore River upstream	8	2.9×10^3	6.3×10^2	9.1×10^3	4.6×10^2	$93 - 1.8 \times 10^3$	
	8	1.7×10^3	1.8×10^2	1.7×10^4	2.2×10^2	$51 - 2.6 \times 10^3$	
Kallang River upstream	15	1.1×10^4	1.9×10^3	5.3×10^5	4.0×10^2	$10 - 4.1 \times 10^3$	
	9	7.1×10^3	2.0×10^3	1.8×10^5	3.3×10^2	$92.8 - 3.3 \times 10^3$	
Geylang River	6	1.3×10^4	2.0×10^2	8.2×10^5	3.4×10^2	$52 - 4.6 \times 10^3$	
Stamford Canal	16	3.3×10^4	4.6×10^3	4.8×10^5	3.0×10^3	$4.9 \times 10^2 - 2.0 \times 10^4$	
Rochor Canal	7	3.6×10^4	1.0×10^4	1.0×10^5	7.8×10^2	$1.2 \times 10^2 - 3.3 \times 10^3$	
Bukit Timah Canal	1		6.8×10^3			487	
Alexandra Canal	1		7.1×10^3			20	
Marina Bay	5	1.2×10^3	2.0×10^2	1.0×10^4	1.2×10^2	$10 - 4.7 \times 10^3$	

The distribution of enteric viruses recovered from surface water samples of Marina Catchment and Bay as determined by nested PCR or RT-PCR is summarized in Table 4.4. The occurrence of enteric viruses in urban surface waters with sampling dates is shown in the appendix. In the present study, adenoviruses, astroviruses, enteroviruses and noroviruses were detected in almost all water catchment and bay areas of interest. Hepatitis A viruses were only detected in water samples collected from the Stamford Canal. At least one enteric virus was detected in 70 of the total 84 surface water samples (83.3%). Three out of six surface water samples collected from bay areas were positive for at least one enteric virus. Notably, in the bay areas, four enteric virus groups (adenoviruses, astroviruses, enteroviruses and noroviruses) were detected in Kallang Basin, which receives water discharge from the Kallang River. Noroviruses GI and GII were the most prevalent viruses detected (detection rate of 54.8 and 69.0%, respectively), followed by astroviruses (50.0%), enteroviruses (40.0%), adenoviruses (35.7%) and hepatitis A viruses (8.9%).

Table 4.4 Presence of enteric viruses in surface water samples collected from Marina Catchment and Bay between June 2006 and January 2009 as determined by nested PCR/RT-PCR

Sampling location	Number of samples collected	No. of virus positive samples/no. tested (%) ^a					
		AdV	AstV	EV	NV GI	NV GII	HAV
Singapore River Upstream	8	3/8 (37.5)	3/8 (37.5)	2/8 (25.0)	2/8 (25.0)	5/8 (62.5)	0/5
Downstream	9	5/9 (55.6)	3/9 (33.3)	2/9 (22.2)	3/9 (33.3)	5/9 (55.5)	0/5
Kallang River Upstream	17	7/17 (41.2)	8/16 (50.0)	3/16 (18.9)	10/17 (58.8)	11/17 (64.7)	0/10
Downstream	11	4/11 (36.4)	6/10 (60.0)	3/10 (30.0)	7/11 (63.6)	8/11 (72.7)	0/6
Geylang River Upstream	2	1/2	1/2	2/2	1/2	2/2	NT ^b
Downstream	5	0/5	2/5	0/5	3/5	3/5	0/4
Stamford Canal	17	7/17 (41.2)	13/16 (81.3)	13/16 (81.3)	13/17 (76.5)	15/17 (88.2)	4/10 (40.0)
Rochor Canal	7	1/7 (14.3)	2/6 (33.3)	3/6 (50.0)	5/7 (71.4)	6/7 (85.7)	0/5
Bukit Timah Canal	1	1/1	0/1	1/1	0/1	1/1	NT
Alexandra Canal	1	0/1	0/1	1/1	0/1	0/1	NT
Marina Bay	6	1/6	2/6	2/6	2/6	2/6	NT
Total	84	30/84 (35.7)	40/80 (50.0)	32/80 (40.0)	46/84 (54.8)	58/84 (69.0)	4/45 (8.9)

^a AdV, adenoviruses; AstV, astroviruses; EV, enteroviruses; NV GI, noroviruses genogroup I; NV GII, noroviruses genogroup II; HAV, hepatitis A viruses

^b NT = non-tested

Figures 4.5, 4.6 and 4.7 show examples of gel electrophoresis of astroviruses, enteroviruses and noroviruses detected in surface water samples by nested RT-PCR assay, respectively. The nested RT-PCR assays yielded readily detectable bands at the predicted fragment size. This showed that non-specific PCR amplification was not a concern with the protocols and the concentrated environmental water samples. Further confirmation of the PCR products by using DNA sequencing is described in Chapter 5.

In order to test the possible PCR inhibition in concentrated environmental water samples, nucleic acid extracted from virus-negative samples was further diluted 10- and 100-fold, and the assay was repeated. Of the 14 virus-negative surface water samples, only one sample (Singapore River downstream, sampling date: 27 December 2006) was positive for norovirus GII after 10-fold dilution of extracted nucleic acid.

According to a binary logistic regression model, the occurrence of adenoviruses, astroviruses, enteroviruses and noroviruses, as determined by nested PCR assays, was not statistically correlated with the levels of *E. coli* ($P > 0.05$). The levels of enterococcus were significantly correlated to the presence of astroviruses ($\chi^2(1) = 7.861$; $P < 0.05$; percentage accuracy = 59.5%), but not significantly correlated to the presence of adenoviruses, enteroviruses and noroviruses ($P > 0.05$).

The estimated concentrations of adenoviruses and noroviruses in surface water samples were determined by quantitative real-time PCR (Table 4.5). Adenoviruses were quantified in water samples collected from catchments and the bay at levels ranging from 10^2 to 10^3 gene copies/liter. The results showed approximately one log higher concentration of noroviruses than adenoviruses in catchment waters, ranging from 10^3 to 10^4 gene copies/liter. To test for possible real-time PCR inhibitors, water samples that were negative for viruses using quantitative real-time PCR were diluted 10- to 100-fold and the PCR assay was repeated. None of these samples were positive for viruses after dilution.

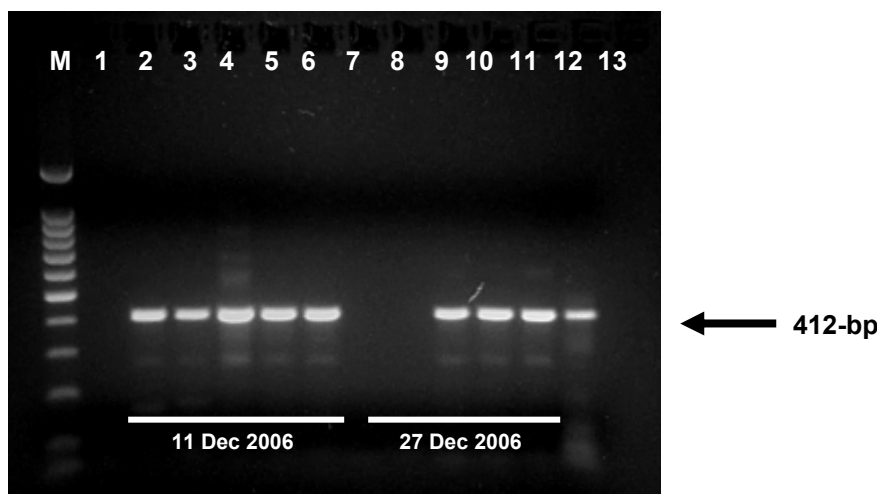


Figure 4.5 Gel photograph of astroviruses detected in catchment water samples (sampling dates: 11 and 27 December 06). Lanes: M, 100-bp DNA marker; 1 and 13: negative control; 2 and 7: Singapore River (downstream); 3 and 8: Singapore River (upstream); 4 and 9: Stamford Canal; 5 and 10: Kallang River (downstream); 6 and 11: Kallang River (upstream); 12: positive control.

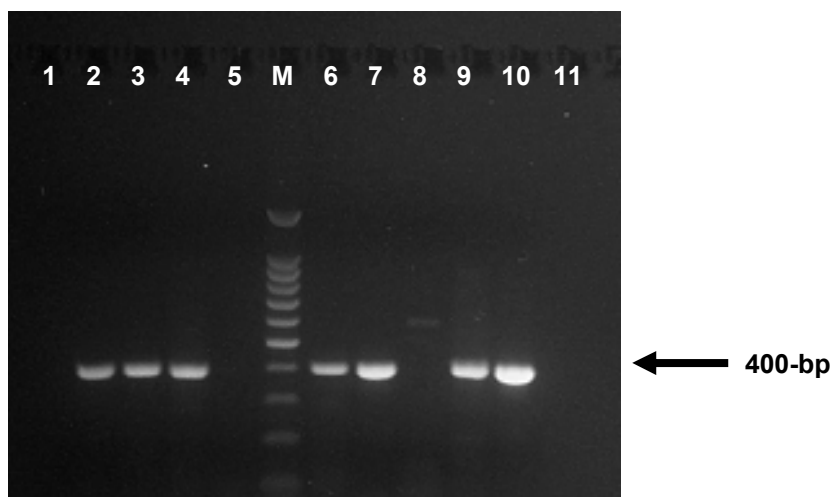


Figure 4.6 Gel photograph of enteroviruses detected in surface waters. Lanes: M, 100-bp DNA marker; 1 and 11: negative control; 2: Marina Bay (14 March 07); 3: Marina Bay (11 April 07); 4: Bukit Timah Canal; 5: Geylang River (downstream); 6: Alexandra Canal; 7: Geylang River (upstream); 8: Singapore River (upstream, 1 November 06); 9: Stamford Canal (1 November 06); 10: positive control.

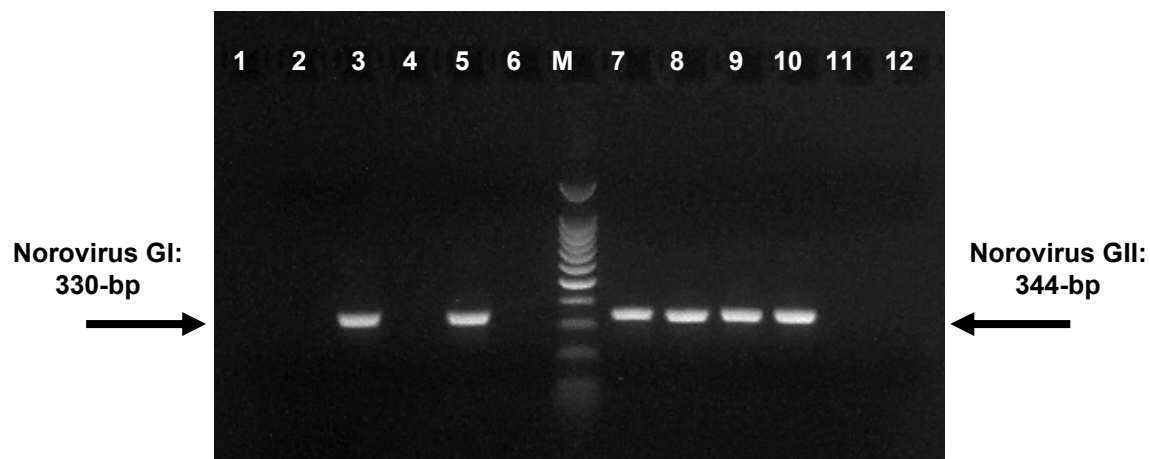


Figure 4.7 Gel photograph of human noroviruses GI and GII detected in catchment water samples (Sampling date: 20 January 2009). Lanes: M, 100-bp DNA marker; 1 to 6, norovirus GI, 330-bp amplicon (1 and 2, negative control; 3 and 5, positive for Stamford Canal and Kallang River, respectively; 4 and 6, negative for Rochor Canal and Geylang River, respectively); 7 to 12, norovirus GII, 344-bp amplicon (11 and 12, negative control; 7-10, positive for all samples tested).

Table 4.5 Estimated concentrations of adenoviruses and noroviruses in surface water samples

Virus	Sampling location	Sampling date	Estimated concentration^{a,b} (gene copy no./liter)
Adenovirus	Singapore River	20 Nov 06	6.8×10^2
		11 Dec 06	3.5×10^2
	Stamford Canal	20 Nov 06	2.4×10^3
		11 Dec 06	4.6×10^3
		17 Oct 08	ND [*]
		03 Nov 08	ND
		26 Dec 08	4.1×10^3
	Rochor Canal	17 Oct 08	6.9×10^2
		03 Nov 08	ND
		26 Dec 08	ND
	Kallang River (Upstream)	20 Nov 06	5.1×10^3
		17 Oct 08	ND
		03 Nov 08	ND
		26 Dec 08	ND
	Geylang River (Downstream)	17 Oct 08	ND
		03 Nov 08	ND
		26 Dec 08	ND
	Marina Bay	14 Mar 07	7.2×10^2
Norovirus GII	Stamford Canal	17 Oct 08	2.4×10^4
		03 Nov 08	1.7×10^4
		26 Dec 08	2.2×10^4
		20 Jan 09	2.3×10^3
	Rochor Canal	17 Oct 08	8.4×10^3
		03 Nov 08	1.3×10^4
		26 Dec 08	ND
		20 Jan 09	2.9×10^3
	Kallang River (Upstream)	17 Oct 08	1.9×10^4
		03 Nov 08	ND
		26 Dec 08	ND
	Geylang River (Downstream)	17 Oct 08	1.0×10^4
		03 Nov 08	2.4×10^4
		26 Dec 08	2.7×10^3
		20 Jan 09	1.6×10^3

^a All water samples were run in triplicate.^b *, ND = non-detected by quantitative real-time PCR

4.2.4 Seasonal detection of bacterial indicator and enteric viruses in urban surface water

During the sampling events in 2006, the seasonal detection of bacterial indicators and enteric viruses was determined. The total rainfall intensity for dry weather (June to August and October) and wet weather (November to December) seasons in catchment areas during the study period was 582.7 mm and 917.4 mm, respectively. An independent-samples t-test was conducted to compare the rainfall intensity for dry and wet weather seasons. There was a significant difference in rainfall intensity for dry weather season (Mean = 145.7, SD = 82.1) and wet weather season (Mean = 458.7, SD = 230.8); $P = 0.006$ (two-tailed). However, there was no significant difference in the levels of *E. coli* and enterococcus for dry and wet weather seasons ($P > 0.05$ for both bacteria) as determined by the independent-samples t-test.

A chi-square analysis indicated that adenoviruses were most frequently detected during the wet weather season than during the dry weather season, $\chi^2 (1, n = 50) = 5.078, P < 0.05$. A similar relationship was observed for the occurrence of astroviruses at the 95% confidence level, $\chi^2 (1, n = 50) = 6.253, P < 0.05$. The seasonal detection rate for enteroviruses, noroviruses GI and GII, however, was not statistically different between the wet and the dry seasons ($P > 0.05$).

4.3 Discussion

4.3.1 Viruses detected in urban wastewaters

The presence of astroviruses, adenoviruses, enteroviruses, hepatitis A viruses and noroviruses in raw sewage and secondary effluent collected from three water reclamation plants was studied by using PCR assays. All enteric viruses of concern were detected in more than 70% of urban sewage although the sample size was small (six months of sampling period). The high prevalence of enteric viruses in sewage suggests the circulation of these viral pathogens at a high frequency among the

population in Singapore. However, this hypothesis could be true only if epidemiological data on these viral pathogens are available on a regular basis. The current study highlights the importance of routine environmental and clinical surveillance of human enteric viruses for better understanding of the actual burden of waterborne viral infections.

Surprisingly, 78% of sewage samples were positive for hepatitis A viruses although Singapore is considered a non-endemic area. However, it is important to note that about 72 % of hepatitis A cases reported in Singapore from Jan to June 2007 were imported (<http://www.moh.gov.sg/mohcorp/statisticsweeklybulletins.aspx>). In developed regions such as Singapore with higher hygiene and advanced water treatment facilities, individuals have less opportunity for infection with enteric pathogens through contaminated food and water and thus, community members are less likely to develop immunity at a young age. This, however, makes them more susceptible to infection upon contact with contaminated water and food. Therefore, hepatitis A virus poses a relatively higher risk for developed regions.

The present study showed the widespread occurrence of all tested enteric virus groups in wastewaters. This is in contrast with the findings of other studies in temperate regions which normally show the prevalence of certain enteric virus group in wastewater samples. In Norway and the United States, Myrmel et al. (2006) and Symonds et al. (2009) showed that adenoviruses were more prevalent in sewage. In another study in France, astroviruses were detected in 90% of raw sewage while noroviruses, hepatitis A viruses and enteroviruses were not detected during the study period (Arraj et al., 2008). A recent study by Petrinca et al. (2009) in Italy identified enteroviruses as the most abundant viruses in sewage among other viruses tested (astroviruses, adenoviruses, hepatitis A viruses, noroviruses and rotaviruses). These discrepancies in enteric viral prevalence could be related to the different epidemiology of viral infection in these different geographical areas.

Reduction of viable viruses in sewage through biological treatment was determined by the direct quantification of coliphages in raw sewage and secondary effluent with plaque assays. The quantitative data of coliphages, adenoviruses, astroviruses and noroviruses in secondary effluent suggests that activated sludge treatment could substantially reduce viruses in wastewater though it could not totally eliminate these pathogens. Astroviruses were detected at the highest concentration in sewage samples throughout the study period among the tested enteric viruses. The viral loads estimated for astrovirus in the present study correspond to levels reported by Le Cann et al. (2004) in France (approximately 10^6 and 10^4 gene copies/100ml for sewage and treated effluent, respectively). Although high numbers of astrovirus genomes were detected in the sewage, a three-log reduction could be achieved with the activated sludge treatment. In contrast, the average removal for noroviruses was lower than that for adenoviruses and astroviruses. The average removal for noroviruses documented in the present study is consistent with the observation in the Netherlands, with 1.8 $\log_{10}$ units by using the activated sludge treatment (Lodder and Husman 2005).

Previous studies have shown that discharges of untreated and treated wastewaters to catchment areas deteriorate quality of drinking and recreational water sources with the presence of enteric viruses (Lodder and Husman 2005; Rodriguez-Diaz et al., 2009). In Singapore, 100% of the population is served by modern sanitation. The treated effluent from the wastewater treatment plants do not feed into the inland waterways, and are discharged out at sea. However, the high prevalence of human enteric viruses in wastewater is significant as these viral pathogens may contaminate drinking and recreational water sources through leaking sewers. Continuous surveillance of human enteric viruses in surface waters is thus warranted to determine the integrity of the sewer system.

4.3.2 Viruses detected in surface waters of Marina catchments and bay

Without natural freshwater resources, the primary water source of drinking water supplies and recreational activities in Singapore is rainfall, collected in water catchment areas or reservoirs. This creates public health concern over possible transmission of waterborne diseases by pathogens present in surface waters particularly enteric viruses. Despite the potential public health hazard posed by enteric viruses, data regarding the occurrence of these potential waterborne pathogens in environmental water sources in tropical countries, such as Singapore, is lacking. In the present study, the prevalence of enteric viruses in surface waters from highly urbanized catchments and the receiving estuarine bay was studied.

The present study demonstrates that tangential flow filtration combined with PCR assay could be an effective method to simultaneously concentrate and detect different groups of human enteric viruses in environmental water samples. Further study is needed to determine the recovery efficiency of tangential flow filtration for concentrating different enteric virus groups simultaneously in environmental waters. A concentration step is critical for detecting pathogenic viruses in environmental waters as their ambient concentrations are usually less than the limit of detection. Ultrafiltration is useful for concentrating different enteric virus groups in a single water sample simultaneously because of the size exclusion-based mode of concentration. Recently, ultrafiltration methods such as tangential flow filtration have shown promise in recovering bacteriophages and polioviruses from environmentally seeded water samples (Morales-Morales et al., 2003; Olszewski et al., 2005; Hill et al., 2007); however, the concentration of different groups of human enteric viruses, including noroviruses, in field samples has not been fully investigated.

Several studies have suggested the use of adenoviruses as an index for human viral contamination in water since they were more frequently detected in water environments (Pina et al., 1998; Lee and Kim 2008b; Hamza et al., 2009). However, the present finding shows that adenoviruses were detected less frequently than astroviruses and both noroviruses GI and GII. One possible explanation for this is that

astroviruses and noroviruses may be shed from the population in Singapore in greater numbers than adenoviruses as shown in the present quantitative results of these viruses in raw sewage.

Although conventional bacterial indicators have helped to protect public health by improving water sanitation, they still suffer from several drawbacks. It has been suggested that bacterial indicators are not accurate microbial indicators of water quality because they survive in sediments, replicate in the environment and can be detected in areas far removed from human activity (Carrillo et al., 1985; Santiago-Mercado and Hazen 1987; Solo-Gabriele et al., 2000; Griffin et al., 2001; Desmarais et al., 2002; Ferguson et al., 2005; Moriarty et al., 2008). In tropical urban ecosystems, nutrients are maintained at high concentrations, and together with constant warm air, soil and water temperatures, this provides an ideal habitat for the survival and growth of *E. coli* (Winfield and Groisman 2003). Moreover, traditional bacterial indicators often fail to predict or correlate with the occurrence of waterborne pathogens of public health concern particularly enteric viruses (Griffin et al., 1999; Jiang et al., 2001; Noble and Fuhrman 2001; Wetz et al., 2004). This is in agreement with the findings in the present study which was conducted in the tropical region. A lack of relationship between bacterial indicators and the presence of pathogenic viruses in water may be partly due to their differences in environmental persistence. It has been demonstrated that environmental factors such as sunlight irradiation, temperature, sediments and nutrients influence the persistence of bacteria and viruses in water environments in different ways (Nasser and Oman 1999; Burkhardt et al., 2000; Allwood et al., 2003; John and Rose 2005; Vinneras et al., 2008).

The high prevalence of human enteric viruses in rivers and canals in Marina Bay area was expected, as there are many old buildings within the catchment and the sewers were laid more than 40 years ago. Many of the sewer systems within catchment areas have deteriorated through age, corrosion and ground settlement (Ooi et al., 2008). Viral contamination in catchment areas may be a result of raw sewage infiltration into waterways via leaking sewer pipes. The current ongoing sewer rehabilitation programme is critical to improve water quality of these urban catchments by

preventing wastewater leakage. The high detection rate of viruses in the urban catchment waters could be due in part, to the stormwater runoff during the wet weather season from November to December.

Enteric viruses were detected in downstream waters of urban rivers and the receiving estuarine bay, indicating urban runoff as a source of viral contamination. The present findings from the field study suggest the environmental stability of enteric viruses in water, which allows them to be transported from the pollution source to the receiving water. The high prevalence of enteric viruses in water environments suggests the possible role played by water as a vector for viral transmission during recreational activities. Studies showing viral infections due to waterborne transmission have been documented, including the ingestion of virus-contaminated water during recreational activities (Gray et al., 1997; Sinclair et al., 2009).

Statistical analysis demonstrated that positive results for astroviruses and adenoviruses were significantly more likely during the wet weather season than during the dry weather season. In contrast, no seasonal occurrence of noroviruses and enteroviruses in water environments was observed. This may suggest that the circulation of enteroviruses and noroviruses in Singapore is not seasonally dependent. However, the seasonal occurrence of enteric viruses in both clinical and environmental samples in the tropical region requires further investigation with more frequent monthly sampling over a year-long period.

The detection of enteric viruses in water samples may serve as a tool to evaluate potential public health risks associated with water usage and current water treatment efficiency. With respect to human health impact, enteric viruses are highly infectious and typically the ingestion of only a few viral particles is necessary to cause infection (Fong and Lipp 2005). These infectious doses can be as few as 10 viral particles or less. For example, the minimum human infectious dose for enteroviruses is on the order of a single virus particle and two- to six-fold lower than the number of enteric bacteria needed to initiate human infection (Metcalf et al., 1995). Therefore, the detection and quantification of enteric viruses in environmental waters is significant for

public health risk assessment. The present study clearly demonstrates that these human pathogens are present in surface waters which serve as important water sources for various recreational activities and potential drinking water supply. However, the overall public health impact can only be determined if the infectivity of enteric viruses recovered from environmental waters is known. Further study is needed to examine the presence of infectious enteric viruses in urban water catchments by using cell cultures. Unfortunately, noroviruses still cannot be cultivated in routine cell culture or in animal models and thus, their infectivity in water environments cannot be determined (Duizer et al., 2004).

A recent study showed that norovirus can be disinfected by adequate free chlorine with provision of proper pre-treatment processes before chlorination (Shin and Sobsey 2008). Virus contamination of drinking water can also be controlled by advanced water treatment processes such as UV and gamma radiation, ozone disinfection and membrane technology (Madaeni 1999; Shin and Sobsey 2003; Husman et al., 2004; Thurston-Enriqueza et al., 2005). Thus, the presence of enteric viruses may represent little public health impact if receiving waters are intended for drinking, especially in areas with advanced water treatment. However, further field studies are needed to evaluate the effectiveness of both conventional and advanced water treatment processes for removing or inactivating human enteric viruses in drinking water. Moreover, adenoviruses have been shown to be more resistant to UV irradiation than other human enteric viruses (Meng and Gerba 1996; Thurston-Enriquez et al., 2003).

4.4 Conclusion

The virological quality of source waters for drinking water supply and recreational activities highly depends on the circulation of particular enteric viruses in the human population. In the absence of specific clinical data on human enteric viruses in Singapore, the present study provides valuable data on the occurrence of potential waterborne viral pathogens in urban surface waters and wastewaters. It has been suggested that pathogen monitoring will be a part of the future characterization of microbiological hazards in water for addressing risk and prevention strategies (Rose and Molloy 2007).

The present study demonstrates that ultrafiltration coupled with molecular techniques could be used as a rapid analytical tool to trace viral contamination of water. Noroviruses were determined to be the most prevalent enteric viruses detected in both wastewater and surface water samples. This finding is significant as noroviruses are the most frequent cause of foodborne and waterborne outbreaks of acute gastroenteritis worldwide. Moreover, noroviruses are one of the major contributors to recreational water illness. A binary logistic regression model indicated that traditional bacterial indicators especially *E. coli* could not be used to adequately predict the presence of enteric viruses in the tropical surface waters.

The multiple barrier approach for providing safe drinking water supply is comprised of source water protection, water treatment processes and maintaining the integrity of water distribution systems. The present study identifies and characterizes potential viral hazards for source water in Singapore. This will enable scientific decisions to be made regarding the selection and prioritization of human pathogenic viruses to be included in the future risk assessment and treatment evaluation for water and wastewater.

Chapter 5

Molecular Characterization of Naturally Occurring Enteric Viruses and Clinical Norovirus Isolates

5.1 Introduction

The application of molecular methods and in particular, the PCR assay has improved the detection of enteric viruses in various environmental water samples. However, the extent of genetic diversity of these viral pathogens, especially noroviruses and astroviruses in sewage and sewage-contaminated surface waters, is poorly understood. Molecular characterization of human enteric viruses isolated from environmental samples may thus yield valuable information on the viral strains circulating in the environment and the community (Bosch et al., 2008).

Traditionally, public health monitoring is conducted at the individual patient level. However, increasing urbanization and high frequency of traveling between different geographical regions necessitate a more proactive public health surveillance (Wong et al., 2007). Surveillance of environmental water samples, particularly community wastewater, can thus be a logical approach to assess the circulation of human pathogenic viruses in a given community and may reveal the occurrence of asymptomatic viral infections. To date, a specific surveillance system and routine laboratory diagnosis for viral gastroenteritis have not been implemented in Singapore and thus, epidemiological data on viral gastroenteritis are limited. It was hypothesized that increases in travel among Asians may contribute to the genetic diversity of pathogenic viruses circulating in the environment and among the population in Singapore.

In addition, molecular characterization of enteric viruses allows the discrimination between animal and human pollution sources. Information on the human and nonhuman origin of fecal pollution could contribute to the public health risk assessment and to the implementation of control measures. The host specificity of enteric viruses suggests that they can be promising library-independent tools to determine the microbial sources of fecal contamination in water environments. It has been suggested that the detection of enteric viruses in waters should include specific viral identification (Mena et al., 2003). This will lead to a more complete viral occurrence database in waters that can be used for scientific decisions regarding urban watershed protection and water and wastewater treatment technologies for viral pathogens removal.

In Chapter 4, the high prevalence of enteric viruses in surface water samples collected from Marina Bay catchments and urban wastewater samples in Singapore was shown. Since environmental water samples may contain multiple virus strains due to accumulation of mutation during replication, each virus-positive sample by the PCR assay was selected for sequence analysis. The DNA sequence analysis is important to further confirm the specificity of PCR assays used in the present study for the direct detection of enteric viruses in complex environmental samples.

Until recently, there have been no reports of molecular characterization of environmental enteric virus isolates in Singapore. The aim of the present study was to provide an understanding of the diversity of individual strains isolated from the tropical aquatic environment and to determine whether some strains are more prevalent in certain areas compared to others. Molecular cloning, DNA sequencing and phylogenetic analysis were used to characterize the enteric virus isolates. Norovirus strains obtained from environmental waters were compared to clinical strains collected from human stools and rectal swabs in the same geographic region during gastroenteritis outbreaks. The comparative study provides valuable information on the possible sources of fecal contamination of surface waters in highly urbanized catchment areas.

5.2 Norovirus genotypes in environmental and clinical samples

5.2.1 Norovirus epidemiology in Singapore

The only reported study of norovirus activity in Singapore was a series of 14 reported outbreaks of norovirus gastroenteritis occurring in December 2003 to January 2004, involving 350 people, and these were linked to oyster consumption (Ng et al., 2005). Singapore General Hospital has been performing norovirus PCR analysis as a routine diagnostic test since 2004 and has observed that increased norovirus activity and outbreaks tend to occur in the second half of the year from July to January (unpublished data). This period coincides with the monsoon seasons (Southwest Monsoon from June to August and Northeast Monsoon from November to January) and inter-monsoon (September to October).

Several outbreaks of gastroenteritis in Singapore occurred during August 2006 to January 2007. There were eight gastroenteritis outbreaks attributed to noroviruses. The outbreaks were from five nursing homes, two schools, and one hospital (a mental health institution). The norovirus-associated gastroenteritis outbreaks in the schools have been documented (Low et al., 2006). The results of molecular characterization of noroviruses isolated during these outbreaks are presented in the following sub-session.

5.2.2 Results

5.2.2.1 Noroviruses in clinical samples during gastroenteritis outbreaks

In the present study, gastroenteritis outbreaks were defined as two or more persons who developed vomiting and/or diarrhea within a two-day period at a location. Samples were collected from residents and staff in nursing home and hospital outbreaks, students and food-handlers in school outbreaks as well as patients in the community. A total of 120 stool and rectal swabs were received during the study period. The clinical specimens were first tested by real-time RT-PCR with TaqMan probes for noroviruses and to determine genogroup (GI or GII). Thirty-five samples

were positive for GII while none was positive for GI. Thirty samples positive for GII were selected for sequencing and of these, twenty-one were sequenced successfully (Table 5.1).

Genotyping of the 21 norovirus-positive clinical samples showed that all of the strains belonged to the GII/4 (genogroup II, type 4). Of the 21 nucleotide sequences isolated from the clinical samples, 20 were identical except for the sample CL4025-0310. The original nucleotide sequences of the clinical norovirus isolates described here have been deposited in the GenBank database under the accession numbers: FJ788330 to FJ788350.

Table 5.1 Characteristics of sequenced norovirus-positive clinical samples

Outbreak / sporadic	Sample code for sequencing	Collection date (day/mo/yr)	Origin	Age (yr) of patient^a	Genogroup/ genotype
Outbreak in 100-bed nursing home	CL4097-3108	31/08/06	Resident	92	GII/4
	CL4098-3108	31/08/06	Resident	84	GII/4
	CL4099-3108	31/08/06	Staff	U	GII/4
Outbreak in 282-bed nursing home	CL4006-1309	13/09/06	Staff	32	GII/4
	CL4007-1409	14/09/06	Resident	85	GII/4
	CL4008-1409	14/09/06	Staff	32	GII/4
	CL4009-1409	14/09/06	Resident	87	GII/4
	CL4010-1409	14/09/06	Resident	79	GII/4
Outbreak in secondary school	CL4015-2909	29/09/06	Food-handler	45	GII/4
	CL4016-2909	29/09/06	Food-handler	42	GII/4
	CL4018-2909	29/09/06	Food-handler	59	GII/4
	CL4021-0310	03/10/06	Food-handler	66	GII/4
Outbreak in mental health hospital	CL4025-0310	03/10/06	Resident	23	GII/4
Outbreak in primary school	CL4029-0310	03/10/06	Student	U	GII/4
	CL4031-0610	06/10/06	Student	U	GII/4
	CL4033-0610	06/10/06	Food handler	67	GII/4
	CL4035-0610	06/10/06	Food handler	68	GII/4
Sporadic	CL4056-1110	11/10/06	Child	6	GII/4
Outbreak in nursing home	CL4095-2010	20/10/06	Resident	76	GII/4
	CL4096-2010	20/10/06	Resident	75	GII/4
	CL4097-2010	20/10/06	Resident	70	GII/4

^a U, Unknown age.

5.2.2.2 Norovirus genotypes in urban surface waters

A neighbor-joining tree constructed from an alignment of the 291-base nucleotide sequence for norovirus GI and the 301-base nucleotide sequence for norovirus GII of the capsid gene revealed multiple genotypes in surface water samples collected from Marina Bay catchment areas (Figure 5.1). Strains belonging to eight different norovirus GI genotypes were identified in urban surface water samples: GI/1, GI/2, GI/3, GI/4, GI/8, GI/9, GI/10, and GI/14. Norovirus GII strains were clustered into 11 genotypes: GII/3, GII/4, GII/6, GII/12, GII/13, GII/14, GII/15, GII/16 and GII/17, and two unclassified genotypes: GII/UG-YURI and GII/UG-Le. Figure 5.1 also shows the genetic relationship between these different norovirus isolates in urban surface waters and reference strains in the GenBank database. All genotype clusters, including two unclassified GII genotypes were statistically supported by bootstrap values of >70%. The nucleotide sequences of noroviruses isolated from surface waters have been deposited in the GenBank database under the following accession numbers: FJ788285 to FJ788329, HM209190 to HM209216 for norovirus GII and FJ788351 to FJ788386, HM209168 to HM209189 for norovirus GI.

The major GI genotypes in surface water samples were GI/2 (15 isolates) and GI/4 (24 isolates). Norovirus GI/2 was not detected during the sampling periods from February 2008 to January 2009. Norovirus GII/4 was the most prevalent GII genotype in surface waters (35 isolates), followed by GII/3 (10 isolates). On the basis of BLAST searches and pairwise comparisons, the most closely related norovirus to the unclassified GII/UG-YURI and GII/UG-Le was the YURI strain, Japan (GenBank accession number: AB083780), with 89.3% nucleotide identity, and the Leverkusen strain, Germany (GenBank accession number: EU424333), with 92.4% nucleotide identity, respectively.

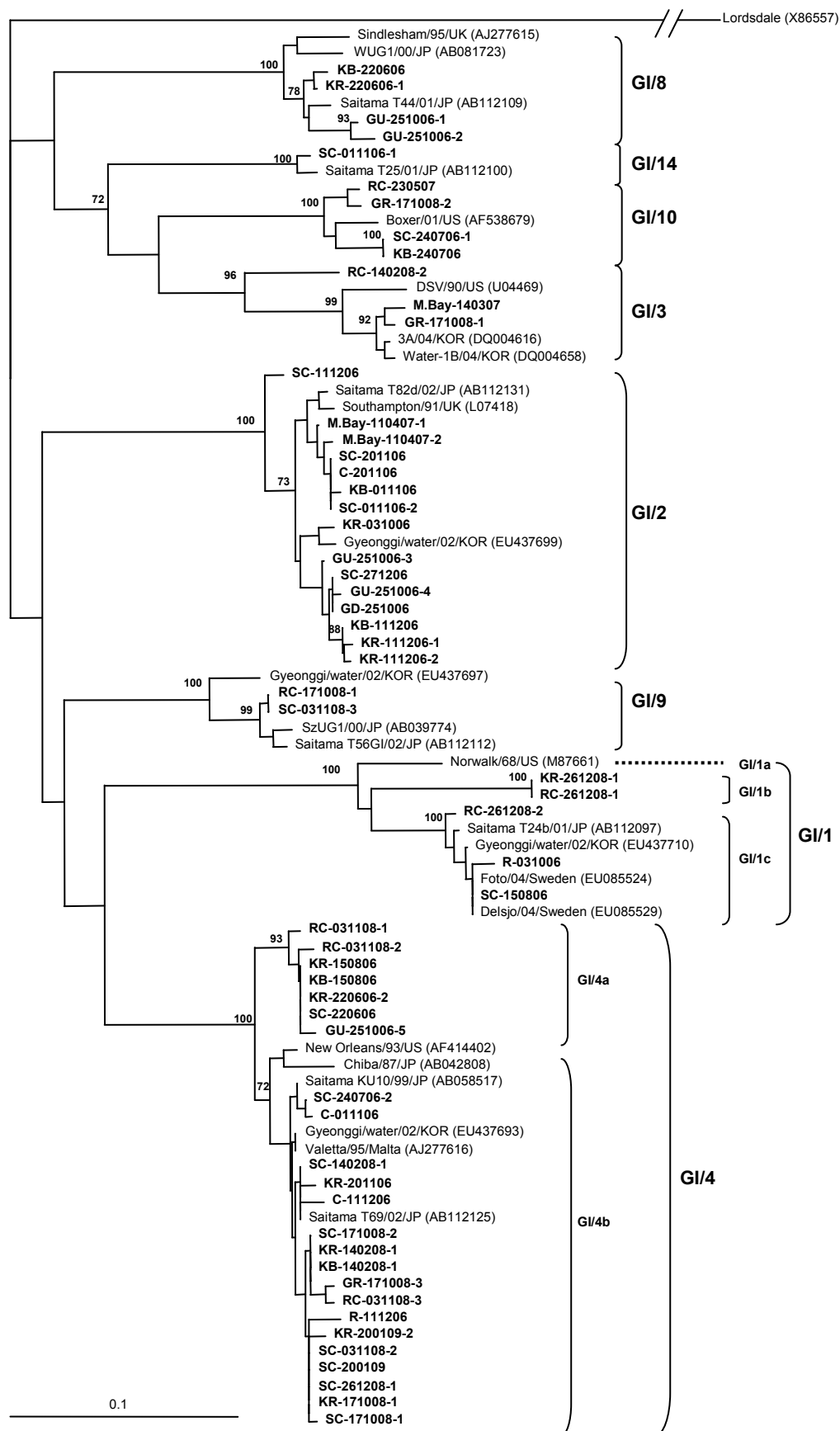


Figure 5.1a (legend on next page)



Figure 5.1b (legend on next page)

Figure 5.1 Phylogenetic tree based on the capsid gene of norovirus, illustrating the genetic relationship of (a) norovirus GI isolates in urban surface waters and the reference strains and (b) norovirus GII isolates in urban surface waters, clinical samples during gastroenteritis outbreaks and the reference strains. The phylogenetic tree was generated by neighbor-joining model with Kimura two-parameter substitution using the PHYLIP package, version 3.67. Numbers at each branch indicate bootstrap values for the clusters supported by that branch. Bootstrap values of ≥ 70 are shown. Nucleotide sequences in the present study are indicated in boldface. Nucleotide sequences of clinical samples are marked with ▲. The number of identical nucleotide sequences obtained from clinical samples is listed in parentheses. When different variants or genotypes were detected within a single sample, they are given the suffixes -1, -2 and so forth.

In the present study, distinct nucleotide sequences could not be identified by direct sequencing of several PCR products recovered from surface water samples. Cloning of these PCR products and sequencing of three to five individual clones resulted in identification of different norovirus genotypes in a single water sample (Table 5.2). These results suggested that cloning of PCR products and sequencing of several individual clones is important, particularly in complex environmental samples, to determine genetic variability of noroviruses.

The norovirus GII/4 strains isolated from surface waters and clinical samples during the same period showed high levels of nucleotide identity (95 to 100%) to each other. A total of 14 environmental isolates (from August to December 2006) in the 2006b cluster were closely related to clinical isolates (Figure 5.1b). Particularly, the nucleotide sequences of six GII/4 environmental isolates (R2U-150806, C1-150806, R1D-150806, C1-011106, R1U-111206, and R1D-111206) and 20 clinical isolates were identical within a 301-bp portion of the capsid gene. In addition, the GII/4 2006b cluster was identified in six environmental isolates in 2007 water sampling events.

Norovirus GII capsid sequences are highly conserved at the N-terminal region and share an identical amino acid start sequence, MKM (Ozawa et al., 2007). In the present study, two environmental isolates were identified as norovirus GII/15. As shown in Figure 5.2, the amino acid start sequence for the viral capsid for this atypical genotype, including the present environmental isolates (KR-140208-1 and SC-261208-1) was MRM, whereas all other noroviruses GII have capsid sequences start with MKM. In addition, an amino acid insertion was observed only in the GII/15 strains within the first 95 amino acids of the capsid gene (Figure 5.2).

Table 5.2 Characteristics of sequenced norovirus-positive surface water samples collected from Marina Catchment and Bay

Water source	Norovirus-positive samples		
	Sample code for sequencing ^a	Genogroup(s) ^b	Genotype(s)
Singapore River	Upstream		
	(Robertson Bridge)		
	R-150806	GII*	GII/4, 12
	R-031006	GI + GII	GI/1, GII/4
	R-011106	GII	GII/4
	R-201106	GII	GII/4
	R-111206	GI* + GII	GI/4, GII/4
	Downstream		
	(Cavenagh Bridge)		
	C-220606	GII*	GII/3
	C-150806	GII	GII/4
	C-011106	GI	GI/4
	C-201106	GI + GII	GI/2, GII/4
Kallang River			
	(upstream)		
	KR-220606	GI*	GI/4, 8
	KR-240706	GII	GII/3
	KR-150806	GI + GII*	GI/4, GII/4
	KR-031006	GI + GII	GI/2, GII/4
	KR-011106	GII	GII/16
	KR-201106	GI* + GII*	GI/4, GII/4 (2 variants), 6
	KR-111206	GI* + GII*	GI/2 (2 variants), GII/6 (2 variants), 14
	KR-271206	GII	GII/4
	KR-111207	GII*	GII/4 (2 variants)
	KR-140208	GI* + GII*	GI/4, GII/UG1
	KR-171008	GI* + GII*	GI/4, GII/3
	KR-261208	GI*	GI/1
	KR-200109	GI* + GII*	GI/4, GII/3
Kallang River			
	(downstream)		
	KB-220606	GI	GI/8
	KB-240706	GI + GII*	GI/10, GII/3, UG2
	KB-150806	GI + GII	GI/4, GII/4
	KB-031006	GII	GII/4
	KB-011106	GI* + GII	GI/2, GII/16
	KB-201106	GII	GII/4
	KB-111206	GI* + GII	GI/2, GII/4
Kallang River			
	(downstream)		
	KB-111207	GII*	GII/4 (3 variants)
	KB-140208	GI* + GII*	GI/4, GII/16

Table 5.2 (Continued)

Water source	Norovirus-positive samples		
	Sample code for sequencing ^a	Genogroup(s) ^b	Genotype(s)
Geylang River			
	Upstream	GU-251006	GI* + GII
Downstream	GD-200607	GII	GI/2 (2 variants), 4, 8 (2 variants), GII/16
	GD-251006	GI + GII*	GII/12
	GD-171008	GI* + GII*	GI/2, GII/16
	GD-200109	GII*	GI/3, 4, 10, GII/3
Stamford Canal	SC-220606	GI + GII*	GII/3
	SC-040706	GII	GII/3
	SC-180706	GII*	GII/3
	SC-240706	GI* + GII	GI/4, GII/UG1
	SC-150806	GI + GII	GII/3
	SC-011106	GI* + GII	GI/4, 10, GII/3
	SC-201106	GI* + GII	GI/1, GII/4
	SC-111206	GI + GII	GI/2, 14, GII/4
	SC-271206	GI + GII	GI/2, GII/4
	SC-140208	GI* + GII*	GI/2, GII/4
	SC-171008	GI* + GII*	GI/4, GII/3
	SC-031108	GI* + GII*	GI/4 (2 variants), GII/4
	SC-261208	GI* + GII*	GI/4, 9, GII/17
	SC-200109	GI* + GII*	GI/4, GII/4 (2 variants)
Rochor Canal	RC-230507	GI + GII	GI/4, GII/4
	RC-140208	GII*	GII/4
	RC-171008	GI* + GII*	GI/9, GII/3
	RC-031108	GI* + GII*	GI/4 (3 variants), GII/14, 16
	RC-261208	GI* + GII*	GI/1 (2 variants), GII/4, 14
	RC-200109	GII*	GII/14 (2 variants)
Bukit Timah Canal	BTC-040407	GII	GII/13
Marina Bay	M.Bay-140307	GI* + GII*	GI/3, GII/4
	M.Bay-110407	GI*	GI/2 (2 variants)
	M.Bay-090507	GII*	GII/4

^a The sample code for sequencing is defined as the sampling location, followed by the day, month, and year of isolation.

^b *, Norovirus genotypes were determined by cloning and sequencing.

	10	20	30	40	50
KR-140208-1	MRMASSDAPV	SGTDGAAGLV	PESQQEVLPL	EPVAGVQLAA	PVAGQSNIID
SC-261208-1					
Hiroshima/JP					
CMH148/THA					
J23/US					
GII/1 Hawaii	K . . . N . . . A P	- N	VNN . TMA .	 ASI .	 LT . . N . V . .
GII/2 Melksham	K . . . N . . . A P	-	 NN . . MA .	 AA . .	 T . . T . .
GII/3 MX	K . . . NR . . A P	- N C . .	 INN . MA .	 AA I .	 LT . . Q . .
GII/4 Lordsdale	K . . . N . . . NP	- DGS . . N . .	 VNN . MA .	 V . AA I .	 Q . V . .
GII/5 Hillingdon	K . . . N . . . TP	- N	 NN . MA .	 V . AS .	 T . . T . .
GII/6 Miami	K . . . N . . . A P	- N N . .	 ANN . MA .	 V . ASI .	 V . Q . .
GII/7 Leeds	K . . . N . . . A P	- N	 INN . M .	 AS . T .	 V . Q . .
GII/8 Amsterdam	K . . . N . . . A P	- N	 INH . MA I .	 AS . .	 V . L . .
GII/12 Honolulu	K . . . N . . . A P	- N	 ANN . TMA .	 ASI .	 LT . . N . .
GII/13 M7	K . . . N . . . TP	- D	 INN . MA .	 AS I .	 V . Q . .
GII/14 Fayetteville	K . . . N . . . A P	- N S . .	 GIN . TM .	 ASI .	 T . .
GII/16 Saitama T53	K . . . N . . . A P	- N . . . T . .	 INT . T .	 AVI .	 T . . T . M .
GII/17 CS-EI	K . . . N . . . A P	- N . . . T . .	 INN . T .	 AA I .	 T . . N . .
	60	70	80	90	
KR-140208-1	PWIRMFVQA	PA SEFTVSPR	NAPGEVLIDL	ELGPELNPLYL	NHLAR
SC-261208-1		. G			
Hiroshima/JP		. G			
CMH148/THA		. G			
J23/US		. G			
GII/1 Hawaii		. NG	. S . . . I . LN .	 F .	. A . . . S .
GII/2 Melksham	 A	. NG	 LN .		. A
GII/3 MX	 MN	. GG	. S LN .	 I	. A
GII/4 Lordsdale	 N	. GG	 I . WSA	P D	. S . . . S .
GII/5 Hillingdon	 T	. NG K .	. S . . . I . VN .		. A
GII/6 Miami	 E	. QG	. S . . . I . LN .		. S . . . S .
GII/7 Leeds	 N	. G	. S . . . I . L .		. A
GII/8 Amsterdam	 N	. G	 F . L .		. A
GII/12 Honolulu	 L	. NG	. S LN .		. A . . . S .
GII/13 M7	 N	. G	. S . . . L . L .		. A
GII/14 Fayetteville	 T	. NG	. S . . . I . LN .	 D	. A . . . S .
GII/16 Saitama T53	 N	. NG	. S . . . I . MN .	 D	. A . . . S .
GII/17 CS-EI	 T	. NG	. S . . . I . LN .	 D	. A . . . S .

Figure 5.2 Multiple alignment of partial deduced amino acid sequences in capsid region of noroviruses GII. The environmental isolates described here are KR-140208-1 and SC-261208-1. Reference strains of GII/15 are Hiroshima/66-1110 (Japan), CMH148 (Thailand) and J23 (United States). Reference strains for other norovirus GII genotypes are also included in this comparison. The predicted highly conserved MKM capsid start sequence is boxed. Dots indicate identical residues.

5.2.2.3 Norovirus genotypes in urban wastewater samples

For wastewater samples, PCR products derived from all norovirus-positive sewage and secondary effluent samples were cloned and at least three colonies were selected for sequencing. In the present study, norovirus strains detected in individual wastewater samples belonged to at least two different genotypes. Five or six norovirus genotypes were also simultaneously detected in individual wastewater samples (Table 5.3). Groups of norovirus GI and GII isolates with 100% nucleotide identity are summarized in the Table 5.4 and Table 5.5, respectively. Only one isolate (representative isolate) from each group was included in the phylogenetic analysis. The nucleotide sequences of noroviruses isolated from urban wastewaters have been deposited in the GenBank database under the following accession numbers: GQ925067 to GQ925162 for norovirus GI and GQ925163 to GQ925246 for norovirus GII.

Over the six-month sampling period, nine different GI genotypes: GI/1, GI/2, GI/3, GI/4, GI/5, GI/9, GI/10, GI/14 and unclassified genotype, GI/UG were detected in urban wastewater samples (Figure 5.3a). The most prevalent GI genotypes were GI/2 (30.3 %) and GI/4 (39.1 %). On the basis of BLAST searches and pairwise comparisons, the most closely related norovirus to the unclassified GI/UG detected in secondary effluent was the Korean strain (GenBank accession number: EU437686) with 95.2 % of nucleotide identity. In Korea, this novel norovirus GI strain was isolated from river water (Lee and Kim 2008a).

Norovirus GII strains in urban wastewater samples were clustered into 10 genotypes: GII/1, GII/2, GII/3, GII/4, GII/6, GII/12, GII/14, GII/16, GII/17 and unclassified genotype, GII/UG-YURI (Figure 5.3b). Norovirus GII/4 (41 %) was the most prevalent GII genotype in wastewater samples. Nine GII isolates (seven isolates from sewage and two isolates from secondary effluent) clustered as unclassified genotype, GII/UG-YURI, were closely related to Japanese norovirus strain, YURI (GenBank accession number: AB083780) with 87.0 to 87.7 % of nucleotide identity. In the present study, the GII/UG-YURI was also detected in surface water samples and shared 90% of nucleotide identity with those isolated from wastewater samples.

Table 5.3 Characteristics of sequenced norovirus-positive wastewater samples collected from water reclamation plants

Sample source	Norovirus-positive samples		
	Sample code for sequencing ^a	Genogroup I	Genogroup II
Bedok WRP			
Sewage (BS)	BS/230107	GI/2, 4 (2 variants), 5, 14	GII/4, 14
	BS/230207	GI/2, 14	GII/4
	BS/200307	GI/3, 4	GII/14, UG
	BS/240407	GI/4, 5, 10	GII/4, UG
	BS/290507	GI/4 (2 variants)	GII/4, 14
	BS/270607	GI/2 (2 variants), 4	GII/4, 16
Secondary effluent (BE)	BE/230107	GI/1, 2, 5	GII/4 (2 variants), 14
	BE/230207	GI/2, 9	GII/3, 6, 14
	BE/200307	GI/5	GII/4 (2 variants)
	BE/240407	GI/2, 4 (2 variants)	GII/16
	BE/290507	GI/2, 4, 5	GII/4 (3 variants), 6
	BE/270607	GI/2, 4	GII/4, 6 (2 variants)
Kranji WRP			
Sewage (KS)	KS/230107	GI/1 (2 variants), 2	GII/4
	KS/230207	GI/4, 5	GII/4 (2 variants)
	KS/200307	GI/2, 4, 5	GII/3, 17 (2 variants)
	KS/240407	GI/4 (2 variants)	GII/UG
	KS/290507	GI/4 (2 variants)	GII/2, 4, 6, UG
	KS/270607	GI/2, 4 (2 variants)	GII/3, 4, 6
Secondary effluent (KE)	KE/230107	GI/1, 2 (2 variants)	GII/4
	KE/230207	GI/2 (2 variants), 4	GII/4, 6
	KE/200307	GI/4, 5	GII/14, 16
	KE/240407	GI/4 (2 variants)	GII/4, 16
	KE/290507	GI/4 (3 variants)	GII/4, 6, 16, UG
	KE/270607	GI/2 (2 variants), 4	GII/4 (2 variants), 16

Table 5.3 (Continued)

Sample source	Norovirus-positive samples		
	Sample code for sequencing ^a	Genogroup I	Genogroup II
Ulu Pandan WRP			
Sewage (US)	US/230107	GI/2, 9 (2 variants)	GII/4, 14
	US/230207	GI/2, 5, 9	GII/1, 4
	US/200307	GI/5	GII/4 (2 variants), UG
	US/240407	GI/4 (3 variants)	GII/6, UG
	US/290507	GI/1, 4 (2 variants)	GII/4, UG
	US/270607	GI/2, 4 (2 variants)	GII/16
Secondary effluent (UE)	UE/230107	GI/2 (3 variants), 4	GII/4, 6
	UE/230207	GI/2, 5 (2 variants)	GII/4 (2 variants), 14
	UE/200307	GI/5 (2 variants)	GII/6, 12, 16
	UE/240407	GI/2, 5	GII/6, UG
	UE/290507	GI/2, 4 (2 variants)	GII/2, 3, 6
	UE/270607	GI/2 (2 variants), UG	GII/3, 4, 6

^a The sample code for sequencing is defined as the sampling location, followed by the day, month, and year of isolation.

Table 5.4 Summary of the characterized norovirus GI strains isolated from the urban wastewaters with 100% nucleotide identity to the representative isolates included in the phylogenetic analysis

Genotype	Representative isolate	Strain(s) with 100% nucleotide identity to the representative isolate
1	KS/230107-1.....	BE/230107-1, KE/230107-2
2	KS/230107-3..... US/230107-2..... BE/230107-2..... UE/240407-1.....	BS/230207-1, KE/230107-1 KE/230107-3, UE/230107-3 UE/290507-2, UE/270607-3 BE/290507-3, BE/270607-1, KE/270607-1
4	KS/230207-1..... BS/200307-2..... BS/240407-2..... US/240407-2..... BS/290507-2..... KS/290507-1..... US/270607-2.....	KE/230207-2 KS/240407-2, US/290507-1, BE/240407-3, BE/270607-2 KS/290507-2, US/270607-1 BS/290507-1 BS/270607-1 BE/240407-2, KE/290507-2, UE/290507-3 KS/240407-1, KE/270607-3
5	BS/230107-2..... KS/230207-2..... BS/240407-1.....	UE/230207-1 US/230207-3, KS/200307-1, UE/200307-2 BE/200307-2, BE/290507-1

Table 5.5 Summary of the characterized norovirus GII strains isolated from the urban wastewaters with 100% nucleotide identity to the representative isolates included in the phylogenetic analysis

Genotype	Representative isolate	Strain(s) with 100% nucleotide identity to the representative isolate
4	BS/230107-1..... KS/290507-2.....	KS/230107-1, BS/230207-1, US/200307-2, BS/240407-1, BS/290507-1, BS/270607-2, BE/200307-2, KE/240407-2, BE/290507-3, KE/290507-3, KE/270607-3 US/290507-1
6	US/240407-2..... KS/290507-1..... UE/230107-2.....	BE/270607-1 UE/290507-3, BE/270607-2 BE/230207-3, KE/230207-1, UE/200307-1
14	BS/200307-1.....	UE/230207-3
16	BS/270607-1.....	BE/240407-1, KE/240407-1
UG-YURI	BS/200307-2.....	BS/240407-2, KS/240407-1, US/240407-1, KS/290507-3, US/290507-2

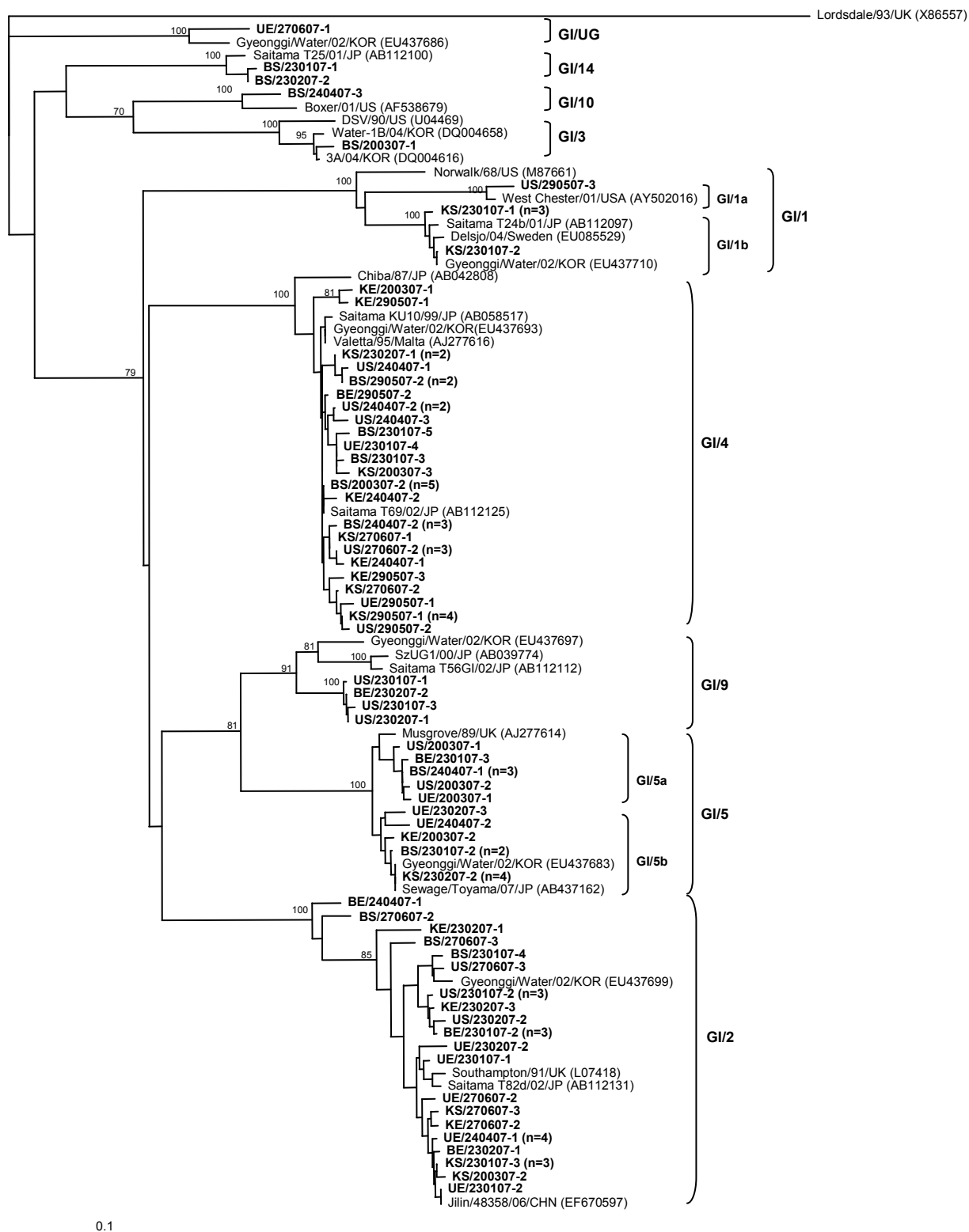


Figure 5.3a (legend on next page)

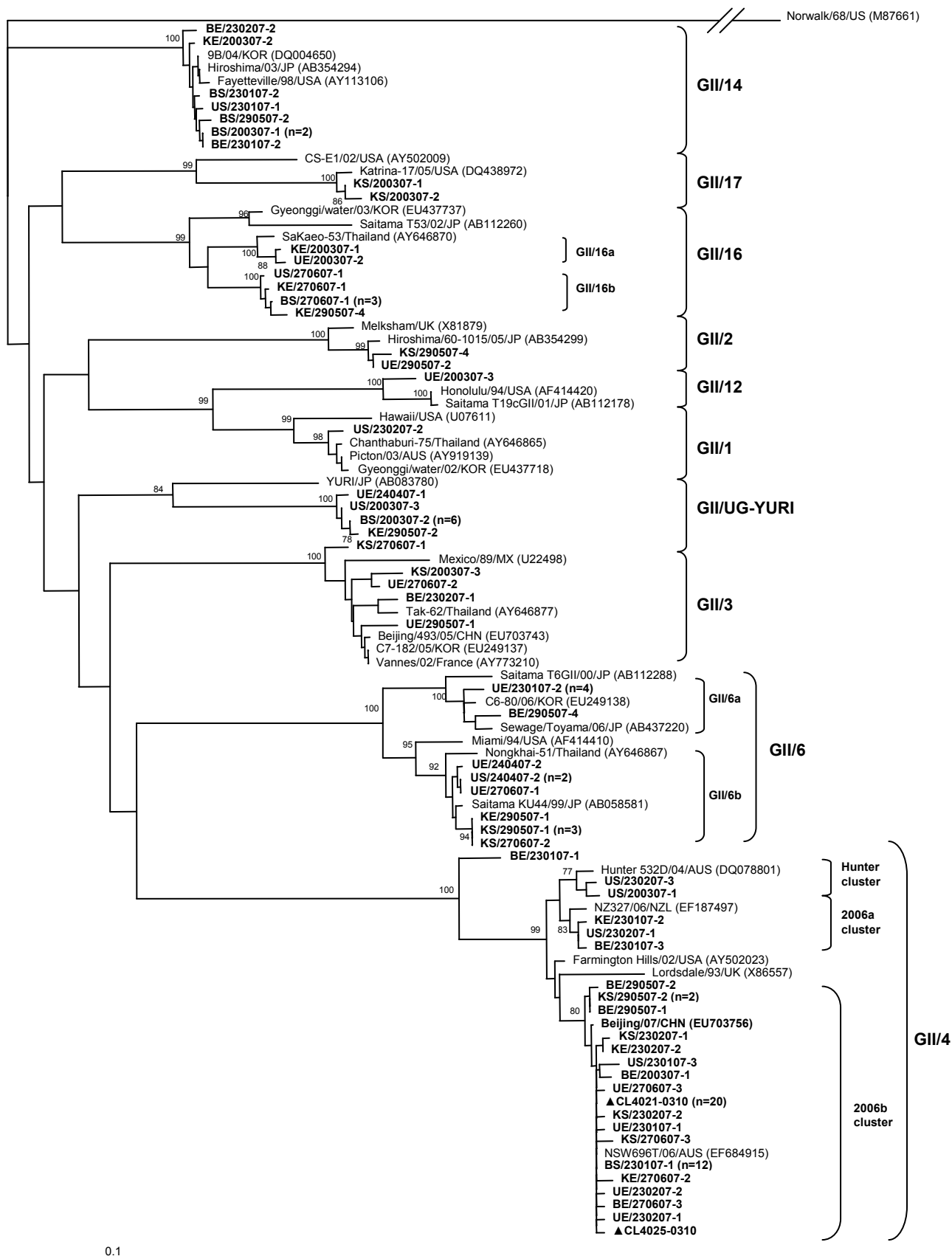


Figure 5.3b (legend on next page)

Figure 5.3 Phylogenetic tree based on the capsid gene of norovirus, illustrating the relationship of (a) norovirus GI isolates in urban wastewater samples and the reference strains and (b) norovirus GII isolates in urban wastewater samples, clinical samples during gastroenteritis outbreaks and the reference strains. The phylogenetic tree was generated by neighbor-joining model with Kimura two-parameter substitution using the PHYLIP package, version 3.67. Numbers at each branch indicate bootstrap values for the clusters supported by that branch. Bootstrap values of ≥ 70 are shown. Nucleotide sequences in the present study are indicated in boldface. Number in parenthesis indicates number of wastewater and clinical samples with the identical sequence detected. Nucleotide sequences of clinical samples are marked with ▲. When different variants or genotypes were detected within a single sample, they are given the suffixes -1, -2 and so forth.

The norovirus GII/4 strains detected in wastewaters showed high levels of nucleotide sequence identity to clinical GII/4 isolates during the gastroenteritis outbreaks in Singapore (Figure 5.3b). In particular, norovirus GII/4 isolates from seven sewage samples and five secondary effluent samples were identical to 20 clinical isolates based on 301-bp of the norovirus capsid region.

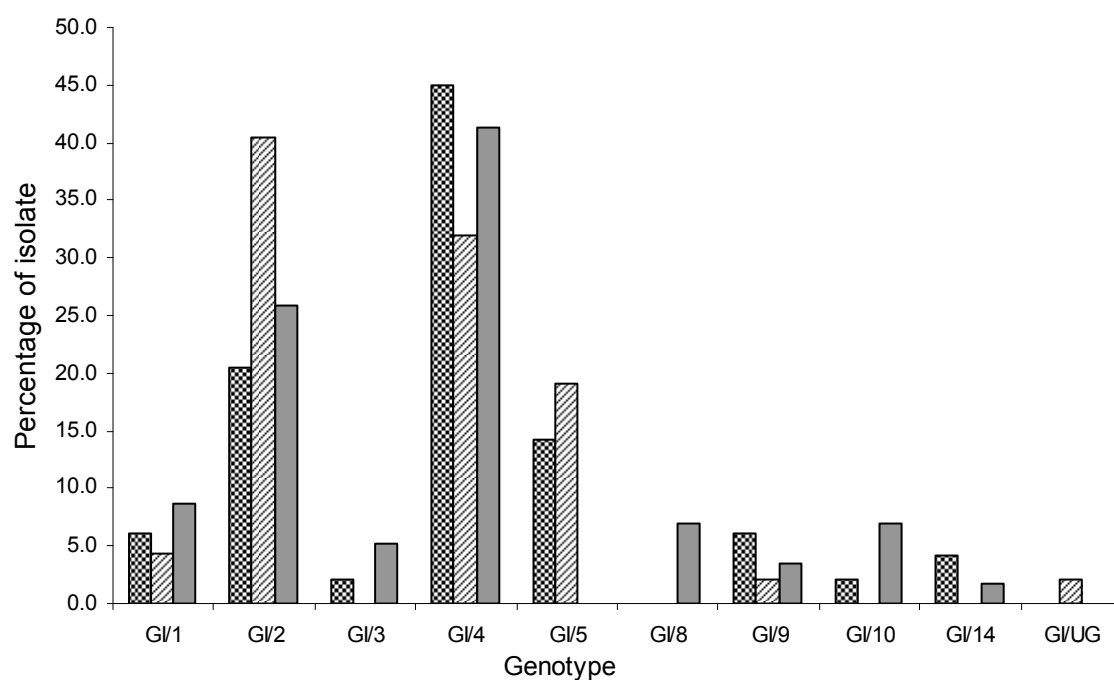
The genetic diversity of noroviruses in sewage, secondary effluent and surface water samples was compared (Figure 5.4). The most prevalent norovirus GI strain in sewage and surface water was GI/4 whereas in secondary effluent, GI/2 predominated. For norovirus GII, the most prevalent genotype in these environmental samples was GII/4. Noroviruses GI/5, GII/1 and GII/2 were only detected in sewage and secondary effluent samples.

5.2.2.4 Comparison of norovirus isolates with others from different geographic locations

Noroviruses isolated from environmental water and wastewater samples in Singapore were compared to strains from different geographic locations. Most of the GI and GII isolates, specifically GI/2, GI/3, GI/4, GI/9, GI/14, GII/1, GII/2, GII/4, GII/6 and GII/16 in the present study were closely related to the reference strains isolated in the Asia Pacific region such as Japan, Korea, China, Australia and New Zealand.

Two GI/1 isolates from surface water samples (R-031006 and SC-150806) and one sewage isolate (KS/230107) showed high nucleotide sequence identity (>98%) to the outbreak strains from Sweden. One clone of water sample collected from Stamford Canal (SC-031108-1) and two clones of sewage sample (KS/200307) showed high nucleotide sequence identity to the GII/17 strain that caused a gastroenteritis outbreak among evacuees of Hurricane Katrina in 2005 in the United States (Yee et al., 2007).

(A)



(B)

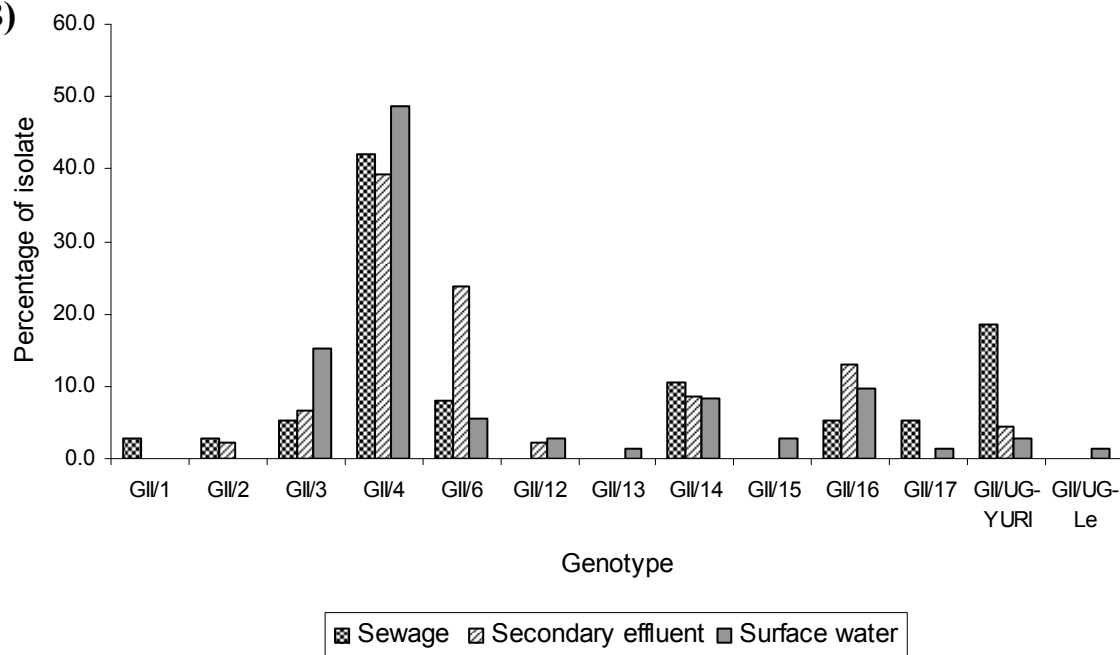


Figure 5.4 Distribution of genotypes of (A) norovirus GI and (B) norovirus GII in surface water, sewage and secondary effluent samples.

Phylogenetic analysis showed that the present GII/4 cluster could be further divided into three small clusters, which were closely related to the Hunter virus and two novel GII/4 variants, 2006a and 2006b from Australia and New Zealand as identified by Tu et al. (2008). These GII/4 reference strains are associated with the global increase in norovirus-associated gastroenteritis epidemics in 2006 (Tu et al., 2008). The GII/4 strains from clinical specimens examined here clustered only with the 2006b variant. A novel GII/4 variant (2008 cluster as shown in Figure 5.1b) was identified in the December 2008 surface water samples.

Besides the GII/4 norovirus, phylogenetic analysis among other norovirus isolates in Singapore and the previously reported reference strains allowed us to distinguish several sub-clusters within each genotype. Sub-clusters of GI/1, GI/2, GI/5, GII/6 and GII/16 were detected in wastewater samples. There was also noticeable sub-clustering within the GI/1, GI/2, GI/3, GI/4, GI/10, GII/3, GII/6 and GII/14 isolates in surface water samples.

Although noroviruses have been increasingly identified as etiological agents that cause waterborne gastroenteritis outbreaks worldwide, relatively few articles have reported the molecular characterization of these viral pathogens in various environmental samples (Table 5.6). The genetic diversity found among environmental norovirus strains in Singapore is in agreement with other Asian and European studies. However, one significant difference between Asian and European studies is that higher degree of genetic diversity of GI norovirus strains was observed in environmental samples in Asian regions, as shown in Table 5.6. Differences in norovirus epidemiology might explain the differences in strains present in the population or in the environment. Moreover, RT-PCR assays targeting different genes used for the detection of noroviruses might contribute to these discrepancies, i.e. the capsid gene was used in the present and other Asian studies whereas RNA-dependent RNA polymerase (RdRp) region was used in European studies.

Table 5.6 Summary of naturally occurring norovirus genotypes isolated from various environmental samples in different geographical regions

Country	Type of sample	Environmental norovirus isolate(s)		Prevalent genotype(s)	Reference
		GI	GII		
The Netherlands	Raw and treated sewage	GI/4, 6	GII/2, 3, 4, 7, 12, unclassified GII types	GI/6, GII/2, GIIb	van den Berg et al. (2005)
The Netherlands	River water	GI/2, 4	GII/3, 4, 7, unclassified GII type	GI/2 and GII/4	Lodder and Husman (2005)
	Raw and treated sewage	GI/2	GII/1, 2, 3, 4, 7, unclassified GII type		
Italy	Raw sewage Seawater	GI/1, 2, 4 Non-detected	GII/3, 4, b GII/4	GII/4	La Rosa et al. (2007)
South Korea	River water	GI/1, 2, 4, 5, 8, 9, 13, unclassified GI type	GII/1, 2, 3, 4, 12, 15, 16	GI/1, 13 and GII/15	Lee and Kim (2008a)
Japan	Raw sewage	GI/1, 2, 4, 5, 8, 11, 12, 14	GII/3, 4, 6, 7, 10, 13, 14, 15	GI/4 and GII/4	Iwai et al. (2009)
Singapore	Urban catchment water (river, canal, estuarine bay)	GI/1, 2, 3, 4, 8, 9, 10, 14	GII/3, 4, 6, 12, 13, 14, 15, 16, 17, unclassified GII types	GI/2, 4 and GII/4, UG-YURI (unclassified GII type)	This study
	Raw and treated sewage	GI/1, 2, 3, 4, 5, 9, 10, 14, unclassified GI type	GII/1, 2, 3, 4, 6, 12, 14, 16, 17, unclassified GII type		

5.2.3 Discussion

The genetic diversity of noroviruses in environmental waters has been reported by others from temperate regions (Lodder and Husman 2005; van den Berg et al., 2005; La Rosa et al., 2007; Lee and Kim 2008a). A recent report from Japan shows that 16 different genotypes of human noroviruses were isolated in sewage samples (Iwai et al., 2009). The present study provides new evidence to show the great genetic diversity of both norovirus GI and GII in tropical water environments and urban wastewaters.

The association of norovirus strains detected in gastroenteritis outbreaks with the total norovirus strains circulating in the community in Singapore is unknown. Therefore, the examination of environmental samples such as urban sewage and surface waters is an opportunity to identify a wider range of strains than might be identified from stool samples in symptomatic cases. Genotyping of 21 clinical samples collected during the same period as the environmental surveys clustered all norovirus strains to GII/4. This is consistent with other clinical molecular epidemiological studies that showed GII, specifically GII/4, as the major causative agent of acute gastroenteritis, especially when associated with outbreaks (Bull et al., 2006; Dey et al., 2007; Ho et al., 2007; Johansen et al., 2008). In contrast, genotyping of environmental water and wastewater samples showed a wide spectrum of genotypes of GI and GII, in addition to the GII/4 strains. The occurrence of multiple genotypes of noroviruses other than GII/4 in environmental samples is important since these strains may be silently circulated in the community after shedding from asymptomatic individuals. These genotypes have been reported as causative agents of gastroenteritis outbreaks (Kageyama et al., 2004), although their isolation from surface water and wastewater samples has rarely been reported. The present results suggest that phylogenetic analysis of both environmental and clinical isolates is a useful approach in understanding the circulation of human noroviruses within the community.

The prevalence of multiple genotypes of GI in water environments corroborates the observations of a study conducted in the urban rivers in Korea (Lee and Kim 2008a). In the Korean study, GI strains were clustered into eight genotypes, with GI/1 and GI/13 as the major genotypes. The majority of GI genotypes in the present study were GI/2 and GI/4. The high prevalence of norovirus GI in both surface water and wastewater samples suggests that several GI strains circulate in the community significantly. Less is known about the occurrence of norovirus GI strains in water environments although GI noroviruses have been identified as etiological agents that cause most of the waterborne norovirus outbreaks (Parshionikar et al., 2003; Maunula et al., 2005; Hewitt et al., 2007). Differences in environmental persistence and routes of transmission between viruses belonging to the two genogroups are possible explanations for GI noroviruses as major causative agents of waterborne outbreaks. However, development of a routine cell culture method is needed for human noroviruses in order to study the environmental stability of both genogroup I and genogroup II.

Two unclassified norovirus genotypes (GI/UG and GII/UG-YURI) could be novel norovirus strains that emerged particularly in the Asian region as they were closely related only to the Korean and Japanese strains. In the present study, the novel GII/UG-YURI strain was detected not only in sewage samples but also in urban catchment waters, suggesting the stability of this norovirus strain in the environment. It is interesting that GII/YURI strain was the second most prevalent norovirus GII isolate in sewage samples in Singapore after GII/4 (Figure 5.4), indicating its widespread circulation in the community during the study period. In addition, most of the environmental isolates of other norovirus genotypes in Singapore appeared more closely related to the clinical strains from Asian countries, especially Japan, Korea, and China. This may indicate that noroviruses in Asia have evolved in a region-specific manner. Our results suggest that noroviruses could be introduced from individuals traveling between geographic regions. Further comprehensive surveillance and phylogenetic analysis of noroviruses in environmental samples should provide valuable information on their circulation and evolution in the populations.

The clinical significance of the environmental norovirus strains was determined by comparing these isolates with the clinical strains obtained during gastroenteritis outbreaks from the same period. Phylogenetic analysis showed that the nucleotide sequences of the norovirus GII/4 strains detected in both clinical and environmental samples were closely related to each other. Moreover, the environmental and clinical norovirus GII/4 isolates (2006a and 2006b clusters) showed the highest levels of capsid alignment with novel GII/4 strains associated with the global gastroenteritis epidemic period in 2006, implying the possible epidemiological relationships of these isolates.

Exposure of individuals to multiple genotypes of noroviruses may lead to mixed norovirus infections, which provide the opportunity for recombination to occur between different strains. It has been shown that recombination is not a rare phenomenon among noroviruses and thus, contributes to the genetic diversity of these viruses (Ambert-Balay et al., 2005). The coexistence of both GI and GII strains in most of the water and wastewater samples, as shown in the present study, may facilitate the emergence of novel intergenogroup recombinants (Phan et al., 2007; Nayak et al., 2008). Discharge of secondary effluent to seawaters and contamination of freshwater through leaking sewers may lead to the exposure of possible hosts to multiple norovirus strains by the use of these water sources for recreation, which may then lead to occurrence of new norovirus recombinants.

The present study highlights the importance of international collaborations and exchange of information on outbreak data, considering that novel norovirus variants are emerging and circulating globally. It has been suggested that global waterborne disease surveillance and reporting networks are needed (Rose and Molloy 2007).

5.3 Enterovirus genotypes in surface water and wastewater samples

5.3.1 Results

DNA sequencing and phylogenetic analysis were used to characterize enterovirus strains present in surface water samples collected from Marina Bay catchment areas and urban sewage samples. The nucleotide sequences of enteroviruses determined in the present study have been deposited in the GenBank database and assigned the accession numbers of GQ925268 to GQ925316 for enteroviruses isolated from sewage and HM209125 to HM209167 for enteroviruses isolated from surface waters.

A phylogenetic tree constructed from the alignment of the 5' non-translated region of enteroviruses isolated from surface waters consisted of two well-separated clades (Figure 5.5a). Human enterovirus C (HEV-C) species formed the first clade. Human enterovirus A (HEV-A) and B (HEV-B) species grouped together as a second clade. A similar genotypes distribution pattern was also observed for the enterovirus-positive sewage samples (Figure 5.5b). Distinct nucleotide sequences could not be identified by direct sequencing of PCR products recovered from all enterovirus-positive sewage samples and several enterovirus-positive surface waters. Cloning of these PCR products and sequencing resulted in identification of different enterovirus genotypes in a single environmental sample (Table 5.7).

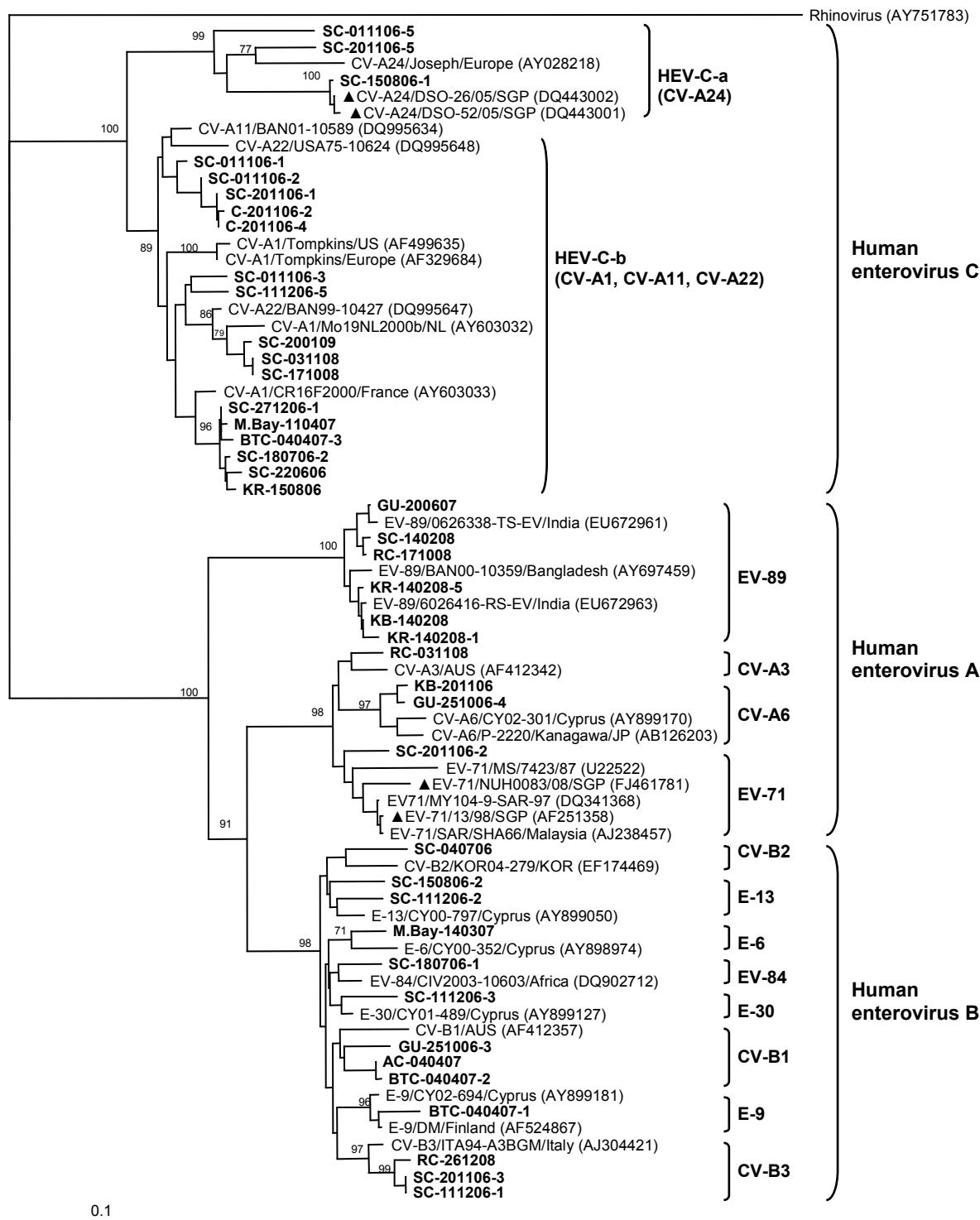


Figure 5.5a (legend on next page)

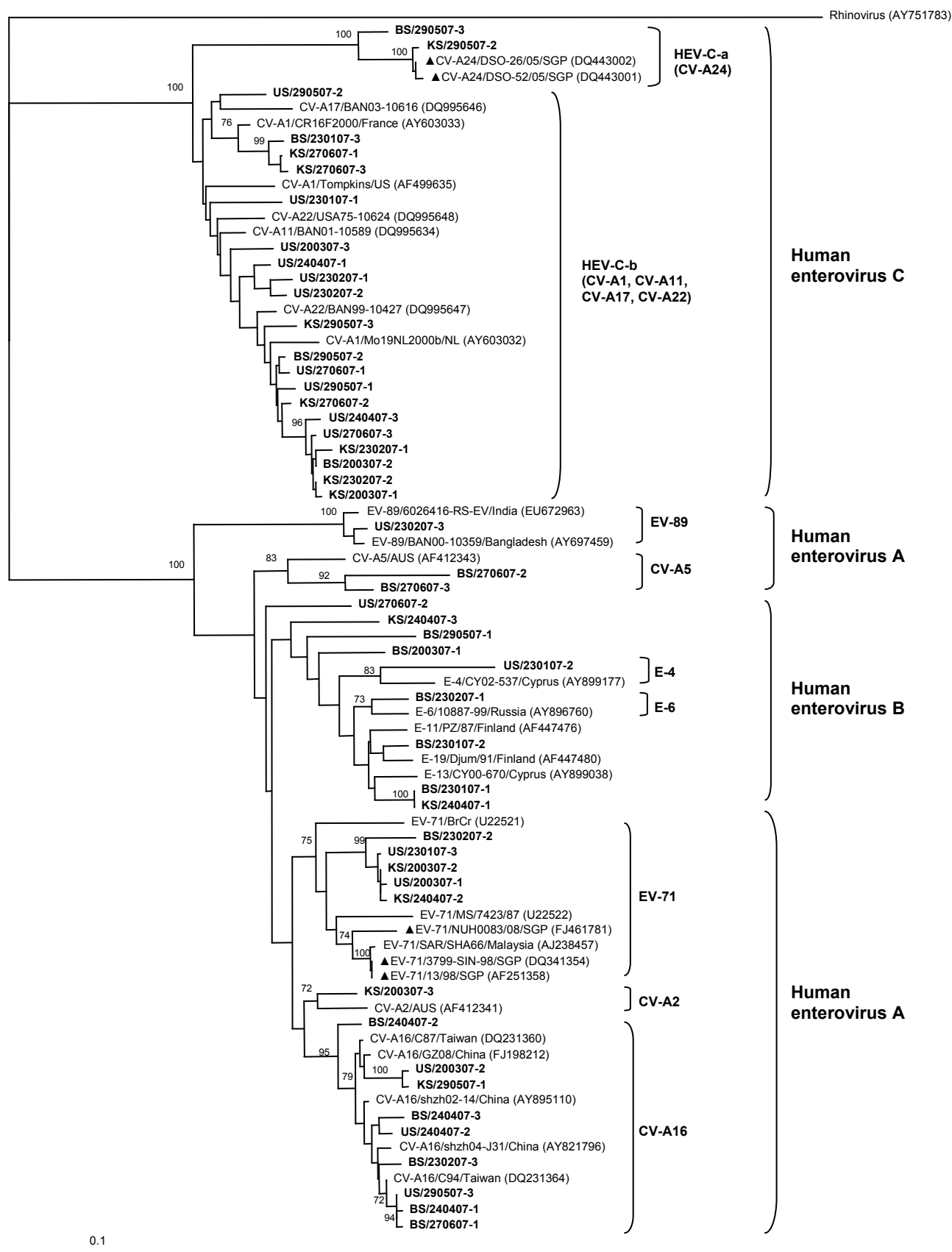


Figure 5.5b (legend on next page)

Figure 5.5 Phylogenetic tree based on partial 5' non-translated region sequences of enteroviruses detected in (a) surface water samples collected from catchments areas of Marina Bay and (b) urban sewage collected from three water reclamation plants in Singapore. The phylogenetic tree was generated by neighbor-joining model with Kimura two-parameter substitution using the PHYLIP package, version 3.67. Numbers at each branch indicate bootstrap values for the clusters supported by that branch. Bootstrap values of ≥ 70 are shown. Nucleotide sequences in the present study are indicated in boldface. Nucleotide sequences of clinical samples isolated during enterovirus-associated outbreaks in Singapore are marked with ▲. When different variants or genotypes were detected within a single sample, they are given the suffixes -1, -2 and so forth.

Table 5.7 Characteristics of sequenced enterovirus-positive surface water and sewage samples

Sample source	Enterovirus-positive samples			
	Sample code for sequencing ^a	EV-A	EV-B ^b	EV-C
Singapore River Downstream (Cavenagh Bridge)	C-201106			CV-A (2 variants)
Kallang River (upstream)	KR-140208	EV-89		
	KR-150806			CV-A
Kallang River (downstream)	KB-201106	CV-A6		
	KB-140208	EV-89		
Geylang River Upstream	GU-251006	CV-A6	CV-B1	
	GU-200607	EV-89		
Stamford Canal	SC-220606			CV-A
	SC-040706		CV-B2	
	SC-180706		EV-84	CV-A
	SC-150806		E-13	CV-A24
	SC-011106			CV-A (3 variants), A24
	SC-201106	EV-71	CV-B3	CV-A, A24
	SC-111206		E-13, 30, CV-B3	CV-A
	SC-271206			CV-A
	SC-140208	EV-89		
	SC-171008			CV-A
	SC-031108			CV-A
	SC-200109			CV-A
Rochor Canal	RC-171008	EV-89		
	RC-031108	CV-A3		
	RC-261208		CV-B3	
Bukit Timah Canal	BTC-040407		CV-B1, EC-9	CV-A
Alexandra Canal	AC-040407		CV-B1	
Marina Bay	M.Bay-140307		E-6	
	M.Bay-110407			CV-A

Table 5.7 (continued)

Sample source	Enterovirus-positive samples			
	Sample code for sequencing ^a	EV-A	EV-B ^b	EV-C
Bedok WRP Sewage (BS)	BS/230107		E-11, 19	CVA
	BS/230207	CVA16, EV71	E-6	
	BS/200307		UT	CVA
	BS/240407	CVA16 (3 variants)		
	BS/290507		UT	CVA, A24
	BS/270607	CVA16, A5 (2 variants)		
Kranji WRP Sewage (KS)	KS/230207			CVA (2 variants)
	KS/200307	CVA2, EV71		CVA
	KS/240407	EV71	E-11, UT	
	KS/290507	CVA16		CVA, A24
	KS/270607			CVA (3 variants)
Ulu Pandan WRP Sewage (US)	US/230107	EV71	E-4	CVA
	US/230207	EV89		CVA (2 variants)
	US/200307	CVA16, EV71		CVA
	US/240407	CVA16		CVA (2 variants)
	US/290507	CVA16		CVA (2 variants)
	US/270607		UT	CVA (2 variants)

^a The sample code for sequencing is defined as the sampling location, followed followed by the day, month, and year of isolation.

^b UT = genotype could not be determined.

HEV-C species were detected in 13 of 17 (76.5%) enterovirus-positive sewage samples and in 15 of 27 (55.6%) enterovirus-positive surface water samples. Within the HEV-C, majority of the field isolates clustered together with coxsackieviruses A1, A11, A17 and A22. Other HEV-C detected was coxsackievirus A24 which was closely related to clinical strains isolated in Singapore during the acute hemorrhagic conjunctivitis outbreak in 2005. HEV-A species (enteroviruses 71 and 89, coxsackieviruses A2, A5 and A16) were detected in 11 of 17 (64.7%) enterovirus-positive sewage samples and HEV-B species were less frequently observed (41.2%) in sewage samples (Figure 5.5b). Nine surface water samples (33.3%) were positive for HEV-A species (enteroviruses 71 and 89, coxsackieviruses A3 and A6) and 10 surface water samples (37%) contained HEV-B species (Figure 5.5a). The HEV-B isolates in surface waters were closely related to enterovirus 84, coxsackieviruses B1, B2 and B3 and echoviruses 6, 9, 13 and 30.

Interestingly, human rhinovirus was identified in a surface water sample collected from Stamford Canal during the December 2008 sampling event (sample code: SC-261208-1, Genbank accession number: HM209167). On the basis of BLAST searches and pairwise comparisons, the most closely related rhinovirus to the present environmental isolate was the novel human rhinovirus C isolated in clinical sample from China (GenBank accession number: GQ223135), with 99.7% of nucleotide identity (Huang et al., 2009). The environmental rhinovirus isolate in the present study shared nucleotide identity of 81% with strains from the United States and Switzerland. The partial 5' non-translated nucleotide sequences alignment of the present environmental rhinovirus isolate and the reference strains is shown in Figure 5.6.

SC-261208-1	10	20	30	40	50	60
NS2/China (GQ223135)	CATGCCGAGGA	ATAGACCTTT	CCAAAGGTTG	AAGCCACAAG	TGTCGTTACC	CACATTGGTA
NAT045/US (EF077280)	T	CC	TAC	T	A	G
CL-170085/SZL (EU840952)	CC	CC	AA	T	T	G
SC-261208-1	70	80	90	100	110	120
NS2/China (GQ223135)	CTACACAGAG	CCTAGTATCG	CTCTAGAAAC	TTGTTGGTTG	GTCGTTCAAGC	CATTTCC
NAT045/US (EF077280)	G	A	T	GT	CC	T
CL-170085/SZL (EU840952)	G	A	TA	CTGA	CTCA	GACCA
SC-261208-1	130	140	150	160	170	180
NS2/China (GQ223135)	CATGGTAGAC	CTGGCAGATG	AGGCAAGAAAG	CTCCCACTG	GTAACAGTGT	TCTTGCCCTGC
NAT045/US (EF077280)	C	A	CT	CA	T	C
CL-170085/SZL (EU840952)	AC	CT	CT	TA	CG	CG
SC-261208-1	190	200	210	220	230	240
NS2/China (GQ223135)	GTGGCTGCCCT	GACACCCCTA	A-TGGGTGT	GAAGCCAATG	AGTGGAACAGG	GTGTGAAGAG
NAT045/US (EF077280)	G	CT	-G	GT	A	A
CL-170085/SZL (EU840952)	CT	CT	TACG	CGA	ACA	A
SC-261208-1	250	260	270	280	290	300
NS2/China (GQ223135)	CCGCGTGTGC	TACCAGTGAG	TCCTCCGGCC	CCTGAATGCG	GCTAATCCAA	CCCCACAGCT
NAT045/US (EF077280)	C	T	A	T	T	T
CL-170085/SZL (EU840952)	C	T	A	T	T	T
SC-261208-1	310	320	330	340	350	
NS2/China (GQ223135)	ATGGCATGCA	ATCCAGCATG	TGCGTAGTCG	TAAATGATAA	TTGTGGGACG	GAACCGAC
NAT045/US (EF077280)	T	CA	TG	ATA	C	T
CL-170085/SZL (EU840952)	G	T	A	AT	C	T

Figure 5.6 Partial 5' non-translated region nucleotide sequence of rhinovirus C isolate in a surface water sample of Stamford Canal (SC-261208-1). The sequence was aligned with the rhinovirus reference strains isolated in clinical samples from China (Genbank accession number: GQ223135), the United States (EF077280) and Switzerland (EU84092). Dots indicate identical residues.

5.3.2 Discussion

Phylogenetic analysis of enteroviruses based on nucleotide sequences located in the 5' non-translated region has been used for rapid identification of enteroviruses in environmental water samples (Shieh et al., 1997; Gersberg et al., 2006; Hsu et al., 2007; Chen et al., 2008b). In the present study, enterovirus isolates separated into two distinct clusters with HEV-C as the first cluster and HEV-A and HEV-B grouped together as the second cluster. This is in agreement with previous studies that used the 5' non-translated region for the phylogenetic analysis of enterovirus isolates (Muir et al., 1998; Siafakas et al., 2002; Clancy et al., 2005). However, members within the HEV-C-b and some HEV-B isolates could not be clearly distinguished with significant bootstrap values. This suggests that there is insufficient sequence variation in the 5' non-translated region to clearly distinguish the enterovirus serotype clusters found in the viral capsid protein 1 (VP1) coding region (Clancy et al., 2005). Partial sequencing of the VP1 protein coding region is needed for future study to better characterize enteroviruses in environmental samples.

The present study revealed the prevalence of coxsackieviruses A (within the HEV-C) in environmental water and wastewater samples. This distribution pattern was not in agreement with the majority of previous studies in temperate regions which showed coxsackievirus B groups (HEV-B) as the dominant enterovirus types in various environmental samples (Hughes et al., 1992; Hovi et al., 1996; Pianetti et al., 2000; Lee et al., 2004; Papaventsis et al., 2005; Costan-Longares et al., 2008; Petrinca et al., 2009). The identification of coxsackievirus B serotypes 1 to 6 has been reported from other studies, although coxsackieviruses B3, B4 and B5 are the most prevalent enterovirus types in the environment (Hughes et al., 1992; Hovi et al., 1996; Pianetti et al., 2000). In contrast, recent studies in Taiwan demonstrated that the predominant serotypes identified in various water systems such as surface water, groundwater and sewage, belonged to the HEV-A (Chen et al., 2008a; Chen et al., 2008b; Hsu et al., 2009). The majority of environmental isolates of HEV-A in Taiwan belonged to coxsackievirus A2, followed by coxsackievirus A6 and enterovirus 71. The high prevalence of coxsackieviruses A of HEV-C in the present study is interesting as this

may suggest a geographical regional difference with respect to enterovirus genotypes or serotypes distribution.

Coxsackieviruses A, particularly within the human enterovirus C, have rarely been detected both in clinical and environmental samples. One reason could be inadequate diagnostic technology as most group A coxsackieviruses grow poorly or produce a discernible cytopathic effect in conventional cell culture (Schmidt et al., 1975; Lipson et al., 1988; Lee et al., 2004). The present study highlights the importance of molecular techniques for the detection and genotypic analysis of rarely identified and difficult-to-cultivate enteric viral strains. It is uncertain why coxsackieviruses A of HEV-C were frequently detected in our environmental samples as the epidemiology of this group of enterovirus in Singapore is poorly understood. However, this new observation may suggest that asymptomatic infections caused by these rare enterovirus strains were widespread in the community during the study period. More recently, Begier et al. (2008) reported an outbreak of meningitis associated with coxsackievirus A1 among a group of travelers after swimming in contaminated seawater in Mexico. However, the contaminated seawater was not tested for enteroviruses during the outbreak. Nevertheless, findings of the report highlight the possibility of direct human exposure to enteroviruses, particularly coxsackieviruses A through recreational activities in surface waters.

Interestingly, seven isolates of enterovirus from environmental samples (six isolates from surface water and one isolate from sewage) was identified as enterovirus 89, a novel enterovirus type within the species human enterovirus A (Oberste et al., 2005). Enterovirus 89 has only been detected in clinical specimens mainly in India (Sapkal et al., 2009) and Bangladesh (Oberste et al., 2005). The detection of enterovirus 89 in different environmental samples suggested the stability of this novel human enterovirus type in water environments.

The identification of human rhinovirus in canal water sample suggests that primers used in the present RT-PCR assay could cross-react with rhinovirus, though these primers have been reported to be specific mainly for enteroviruses (Hafliger et

al., 1997; Chen et al., 2008b). Human rhinovirus is a major cause of upper respiratory tract infections or common colds worldwide (Mackay 2008). The primary routes of transmission are via aerosols of respiratory droplets and from contaminated surfaces. Human rhinoviruses have been genetically classified into two species, human rhinovirus (HRV)-A and HRV-B (Savolainen et al., 2002). More recently, the existence of other species (HRV-C and HRV-D) has been suggested as some genomic sequences are not clustered with HRV-A and HRV-B species (Kiang et al., 2008; Palmenberg et al., 2009). The present environmental HRV-C isolate was closely related to the clinical strains isolated from China based on sequences of the 5' non-translated region (Huang et al., 2009). It is uncertain why rhinovirus was present in the water environment. One possible explanation could be that human rhinoviruses derived from respiratory secretions were carried into the canal by discarded used tissues or leaking sewage. More recently, Blomqvist et al. (2009) reported for the first time, the isolation and identification of HRV-A in sewage specimens collected from Finland, Latvia and Slovakia.

Human coxsackievirus A24 isolated from environmental samples in the present study were closely related to the strains that caused the acute hemorrhagic conjunctivitis outbreak in Singapore in 2005 (Yeo et al., 2007). The detection of coxsackievirus A16 and enterovirus 71 in sewage samples collected from February to June 2007 was correlated with the epidemic period of hand, foot and mouth disease (HFMD) in Singapore (Ang et al., 2009). Coxsackievirus A16 was the predominating strain circulating during the epidemic in 2007. However, coxsackievirus A16 was not detected in surface water samples over the period of study. This may suggest that coxsackievirus A16 is less stable in surface water environments. Unfortunately, nucleotide sequences of coxsackievirus A16 during the HFMD epidemic period are not available and thus, a comparative study between clinical and environmental viral isolates could not be conducted.

5.4 Astrovirus genotypes in surface water and wastewater samples

5.4.1 Results

In total, 85 nucleotide sequences of astrovirus isolated from environmental samples (47 isolates from wastewater samples and 38 isolates from surface water samples) were included in the phylogenetic analysis. Phylogenetic tree was constructed, on the basis of the 348-bp nucleotide sequences from the ORF2 region of astrovirus isolates, to study the genetic relationship between the environmental astrovirus isolates and other published sequences (Figure 5.7). Pairwise comparisons demonstrated groups of environmental human astrovirus type 1 (HAstV-1) isolates in Singapore with 100% nucleotide sequence identity. These groups of isolates and representative isolates of each group are summarized in Table 5.8 according to their sample type. Only the representative isolate from each group was included in the final phylogenetic tree. The nucleotide sequences of astroviruses determined in the present study have been deposited in the GenBank database and assigned the accession numbers of GQ925317 to GQ925363 for astroviruses isolated from wastewater samples and HM209087 to HM209124 for astroviruses isolated from surface water samples.

Human astrovirus types 1, 2, 4, 6 and 8 were identified in surface water samples collected from catchment and bay areas. Three genotypes of human astroviruses were only identified in urban wastewater samples: HAstV-1, HAstV-2 and HAstV-8. Overall, HAstV-1 was the most prevalent type and found in almost all environmental samples (74 out of 85 isolates, 87.1%) over the study period. HAstV-8 was identified in 5.9% of the total environmental isolates. HAstV-2 and HAstV-4 were each identified in two environmental isolates. HAstV-4 was only detected in surface water samples collected from the estuarine bay area. HAstV-6 and feline astrovirus was identified in surface water sample collected from the Stamford Canal (sample code: SC-200109) and Rochor Canal (samples code: RC-031108), respectively. The feline astrovirus isolated from the canal water was closely related to the strain

previously isolated from the United Kingdom (GenBank accession number: AF056197), with nucleotide identity of 85.3%.

Table 5.8 Summary of the human astrovirus type 1 (HAstV-1) isolated from environmental samples with 100% nucleotide identity to the representative isolates included in the phylogenetic analysis

Sample type	Representative isolate	Strain(s) with 100% nucleotide identity to the representative isolate
Surface water	R-031006	KB-031006, KR-031006, C-111206, SC-271206-5
	C-201106	R-201106, SC-201106, KR-201106, SC-111206, KR-111206, GU-251006, GD-251006, SC-271206-1, KR-171008
	KR-011106	KB-201106
	KB-271206	KR-271206
Urban sewage	BS/230107	KS/230107, US/230107, KS/230207, US/230207, US/240407-1, US/290507, BS/270607-2
	BS/230207-2	US/270607-4
	BS/200307	KS/200307, US/200307
	KS/270607-2	US/270607-1
Secondary effluent	BE/230107	KE/230107, UE/230107, BE/230207, KE/230207, UE/230207, BE/270607-2, KE/270607
	BE/200307	KE/200307, UE/200307, BE/290507, UE/290507, UE/270607
	BE/240407	UE/240407

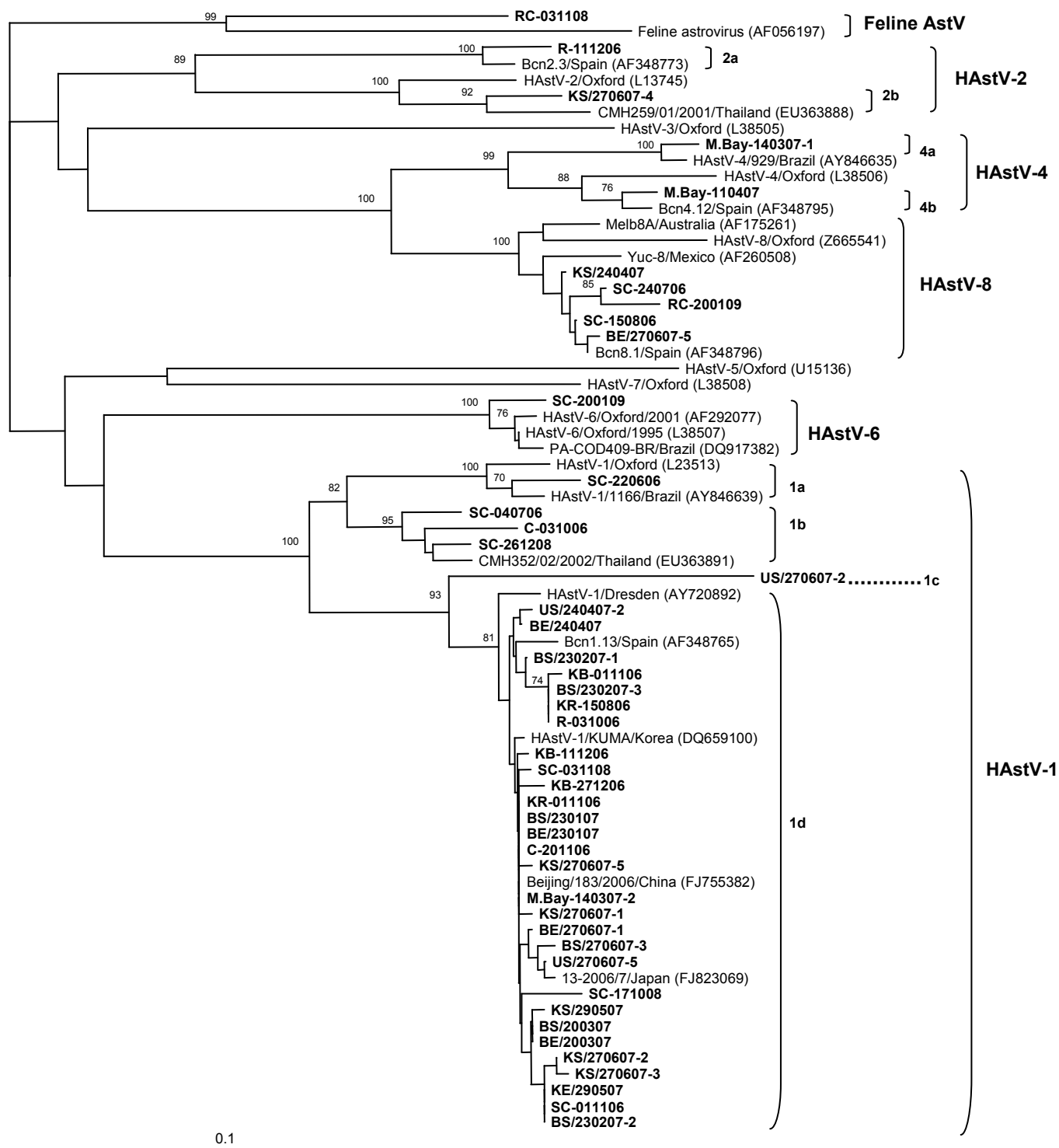


Figure 5.7 (legend on next page)

Figure 5.7 Phylogenetic tree of a 348-bp region of ORF2 of astrovirus environmental strains isolated from surface water, urban sewage and secondary effluent samples in Singapore. Standard human astrovirus strains isolated from the United Kingdom (Oxford strains) and other reference strains in the GenBank database are included in this phylogenetic analysis. The phylogenetic tree was generated by neighbor-joining model with Kimura two-parameter substitution using the PHYLIP package, version 3.67. Numbers at each branch indicate bootstrap values for the clusters supported by that branch. Bootstrap values of ≥ 70 are shown. Nucleotide sequences in the present study are indicated in boldface. When different variants or genotypes were detected within a single sample, they are given the suffixes -1, -2 and so forth.

In the present study, distinct nucleotide sequences of astrovirus could not be identified by direct sequencing of eight PCR products recovered from environmental samples. Cloning of these PCR products and sequencing of five individual clones resulted in identification of different astrovirus variants in a single sample. In particular, two different serotypes of astrovirus were simultaneously detected in samples M.Bay-140307 (HAstV-1 and -4), KS-270607 (HAstV-1 and -2) and BE-270607 (HAstV-1 and -8). Two to five variants of HAstV-1 were identified in samples SC-271206, BS-230207, US-240407, BS-270607, KS-270607, US-270607 and BE-270607.

The degree of variation was evident between Singaporean astrovirus isolates and prototype Oxford strains from the United Kingdom. Singaporean HAstV 1, 2, 4, 6 and 8 isolates displayed 3.5 to 16.4%, 6.5 to 14.3%, 4.6 to 8.4%, 2.1% and 5.9 to 7.3% nucleotide sequence variation, respectively with the nucleotide sequence of the Oxford strains. Furthermore, the phylogenetic analysis showed that HAstV-1 could be grouped into four lineages (1a to 1d) (Figure 5.7). The majority of the environmental HAstV-1 isolates in Singapore clustered into the lineage 1d, together with other clinical isolates from Germany, Spain, Korea, China and Japan. Within HAstV-2 and HAstV-4 isolates in the present study, only two major lineages were identified. No major lineages could be clearly discriminated within HAstV-8 isolates. The present environmental HAstV-8 isolates were closely related to the clinical isolates from Mexico and Spain.

5.4.2 Discussion

Human astroviruses have been increasingly identified as important etiologic agents of viral gastroenteritis in all age groups (Glass et al., 1996; Mitchell et al., 1999). However, only a few studies have characterized the astroviruses isolated from environmental water samples (Nadan et al., 2003; Meleg et al., 2006; Rodriguez-Diaz et al., 2009). In the present study, astroviruses isolated from surface water and wastewater samples were characterized according to the nucleotide sequences of a 348-bp fragment of the ORF2 region. It has been shown that there is a good correlation

between astrovirus antigenic and genomic types based on this capsid gene region (Noel et al., 1995).

The molecular characterization of environmental astrovirus isolates revealed the prevalence of HAstV-1. This is in agreement with previous clinical studies worldwide which reported genotype 1 as the predominant disease-causing astrovirus type (Shastri et al., 1998; Mustafa et al., 2000; Guix et al., 2002; Gabbay et al., 2007; Nguyen et al., 2008). A study by Nadan et al. (2003) in South Africa demonstrated that HAstV-1 to 5, -7 and -8 could be isolated from sewage samples, with HAstV-1 as the prevalent astrovirus type. Findings of the South African study also indicated that the human astroviruses detected in both clinical and environmental samples were closely related. Unfortunately, the molecular epidemiology of astrovirus infections has not been studied in Singapore and thus, nucleotide sequences of clinical astrovirus isolates are not available for comparative analysis. In addition, an environmental study of human astroviruses in Hungary demonstrated that HAstV-1 was widely circulated in community sewage samples (Meleg et al., 2006). In contrast, a recent study from South America revealed that all environmental astrovirus isolates belonged to HAstV-8 (Rodriguez-Diaz et al., 2009).

In the present study, uncommon (HAstV-2 and HAstV-4) and rare (HAstV-6 and HAstV-8) human astrovirus genotypes were also detected in environmental samples. It was noted that HAstV-8 isolates in Singapore showed 100 % similarity to most HAstV-4 isolates in the GenBank database at the amino acid level. This is in agreement with a clinical study conducted in Barcelona, Spain, showing a close relationship in this capsid region (Guix et al., 2002). Interestingly, HAstV-6 was detected in a surface water sample collected from an urban canal during the latest sampling event of the study (January 2009). To our knowledge, this is the first study that reveals the presence of this astrovirus genotype in the water environment. Further occurrence study is needed to determine the continuous existence of HAstV-6 in water environments.

The identification of feline astrovirus in the present study was not expected as primers used in the RT-PCR are reported to be of human origin (Oh et al., 2003). Marina catchment areas are highly urbanized and there are no animal farms around the sampling locations. However, fecal matter from pets such as cats and dogs could be the source of fecal contamination in urban water catchments. An infection risk of humans by animal astroviruses in water sources is poorly understood. Further study is needed to detect astroviruses of animal origin in water environments by using RT-PCR assays specific for animal astroviruses. This will improve our understanding of the occurrence of animal astroviruses in water environments and their role of zoonotic infections.

5.5 Adenovirus and hepatitis A virus genotypes in surface water and wastewater samples

5.5.1 Results and discussion

Molecular characterization of adenoviruses was performed by sequencing 16 out of 30 detected strains in surface water samples. The sequencing data generated was compared to the sequences of adenovirus deposited in the GenBank database and showed that 15 of the strains belonged to species F, enteric adenovirus type 40 (4 isolates) and 41 (11 isolates). One environmental adenovirus isolate was identified as human adenovirus type 12, with 100% nucleotide sequence identity to the reference strain (GenBank accession number: X73487) based on 120-bp hexon gene. For sewage samples collected from water reclamation plants, 10 adenovirus-positive samples were sequenced. All sequenced sewage adenovirus isolates were identified as adenovirus type 41, with 98.8 to 99.6% nucleotide identity to the reference strain Tak (GenBank accession number: X51783). The nucleotide sequences of adenoviruses determined in the present study have been deposited in the GenBank database and assigned the accession numbers of GQ925258 to GQ925267 and HM209217 to HM209232. Sequencing results of the environmental adenovirus isolates in Singapore are summarized in Table 5.9.

Table 5.9 Characteristics of sequenced adenovirus-positive surface water and sewage samples

Sample source	Adenovirus-positive samples	
	Sample code for sequencing ^a	Serotype
Surface water		
Singapore River		
Upstream (Robertson Bridge)	R-011106	41
Downstream (Cavenagh Bridge)	C-220606	40
	C-180706	40
	C-111206	41
Stamford canal	SC-220606	41
	SC-180706	40
	SC-011106	41
	SC-111206	12
Kallang River (downstream)	KB-031006	41
	KB-011106	40
Kallang River (upstream)	KR-150806	41
	KR-031006	41
	KR-011106	41
Geylang River (upstream)	GU-251006	41
Bukit Timah Canal	BT-040407	41
Marina Bay	M.Bay-140307	41
Sewage		
Bedok WRP	BS/230107	41
	BS/200307	41
	BS/290507	41
	BS/270607	41
Kranji WRP	KS/290507	41
	KS/270607	41
Ulu Pandan WRP	US/230107	41
	US/200307	41
	US/290507	41
	US/270607	41

^a The sample code for sequencing is defined as the sampling location, followed followed by the day, month, and year of isolation.

The high prevalence of adenoviruses type 40 and 41 in water environments is significant as these enteric adenoviruses tend to survive longer than other enteric viruses in surface waters, representing a potential route of viral transmission (Enriquez et al., 1995). In addition, enteric adenoviruses have been demonstrated to be more resistant to UV light disinfection in drinking water treatment processes (Meng and Gerba 1996; Thurston-Enriquez et al., 2003; Nwachuku et al., 2005; Baxter et al., 2007). The present finding is in agreement with previous studies which demonstrated that the majority of human adenoviruses in surface waters and sewage samples were enteric strains (Van Heerden et al., 2005; Haramoto et al., 2007). The molecular characterization of adenoviruses in water environments is important for risk assessment of infection and burden of diseases associated with adenoviruses in water supplies. Enteric adenoviruses cause gastroenteritis mainly in children and the faecal-oral route is a predominant mode of their transmission. This is different from other serotypes of adenoviruses which cause respiratory and eye infections in all age groups and which are not generally transmitted by the faecal-oral route (Hierholzer 1992; Jiang 2006).

In order to confirm the specificity of the hepatitis A virus (HAV) detection in environmental samples, nested PCR products from the 5' non-translated region of four positive water samples collected from Stamford Canal (sampling date: 11 and 27 December 2006, 26 December 2008 and 20 January 2009) and five positive secondary effluent samples were sequenced. Sequence analysis of the 5' non-translated region of the HAV showed all isolates were identical and closely related to the clinical HAV strains isolated from China, Thailand and South Africa, with more than 99% nucleotide identity. The VP1/2A junction region (viral protein region) is recognized as one of the most variable regions of the HAV genome and is therefore suitable for genotyping (Robertson et al., 1992). The sequence from the VP1/2A region was determined for six HAV-positive sewage samples. Sequence comparison of six Singaporean isolates with other isolates in the GenBank database confirms the presence of HAV strains belonging to genotype IA. The original nucleotide sequences of the environmental HAV isolates described here have been deposited in the GenBank database under the accession numbers of GQ925247 to GQ925257 and HM209233 to HM209236.

The present genotype IA environmental isolates were closely related to common laboratory reference strains LA and HAS-15 isolated from the United States (Robertson et al., 1992). Neighbor-joining tree derived from an alignment of 398-bp of the VP1/2A fragment revealed that the Singaporean HAV isolates were significantly similar to clinical strains isolated in Asia (Figure 5.8). Four sewage samples showed the presence of HAV genotype IA closely related to strains isolated from Indonesia (nucleotide sequence identity from 98.7 to 99.7%). The other two sewage isolates were 98.9% sequence identical to Japanese strains. Point mutation was observed in the nucleotide sequence of the VP1/2A junction region of the present HAV isolates which resulted in an amino acid change. The G at the position of the Indonesia-like isolates is changed to a C in the Japan-like isolates. This mutation results in the substitution of the valine to leucine. HAV genotype IA appears to be responsible for the majority of hepatitis A cases worldwide, whereas genotype IB viruses have been mainly detected in locations near the Mediterranean region (Robertson et al., 1992).

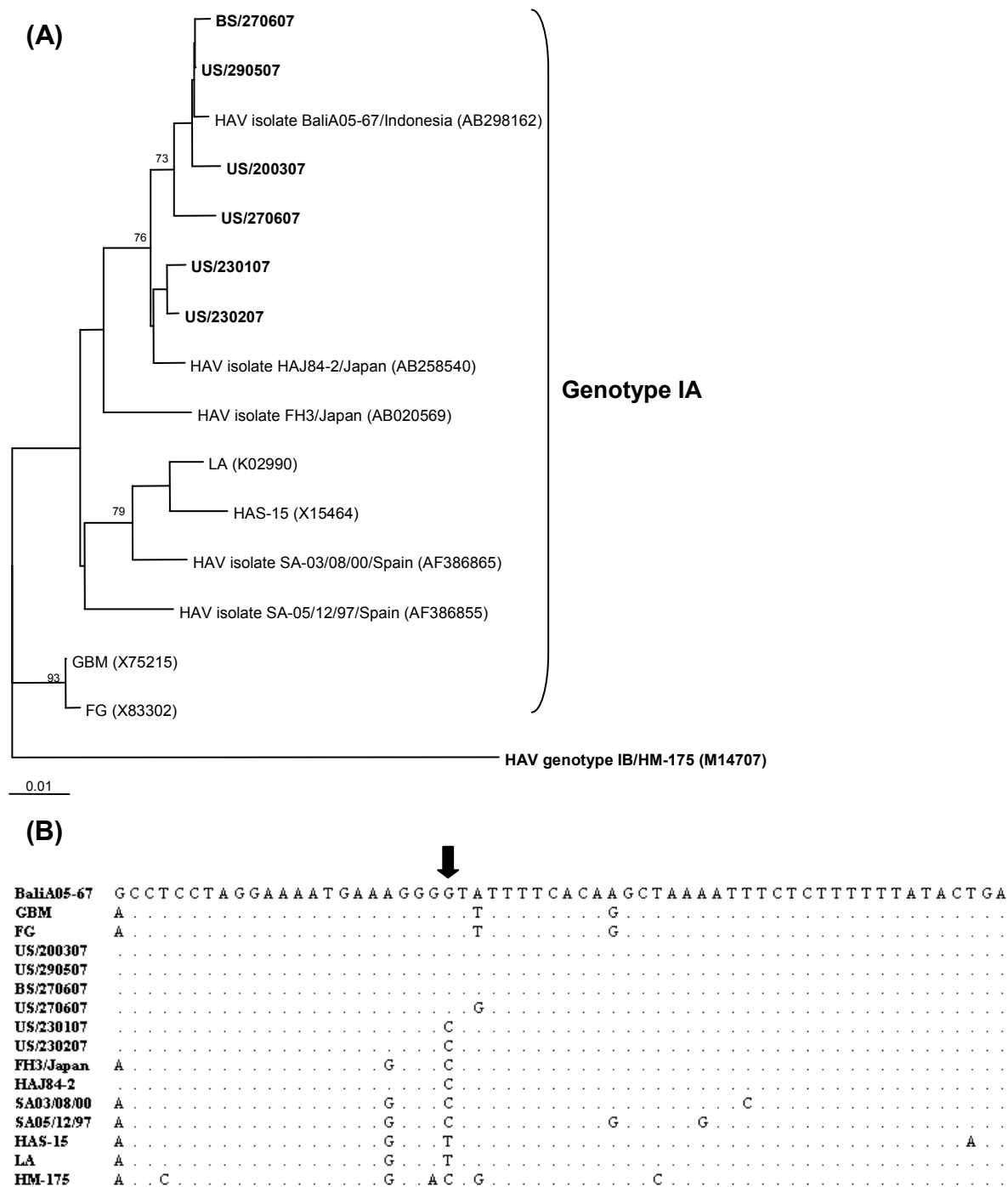


Figure 5.8 (A) Neighbor-joining tree constructed to represent phylogenetic relationships of HAV detected in urban sewage samples collected in Singapore and other reference HAV strains previously reported in other geographic regions. Nucleotide sequences in the present study are indicated in boldface. (B) Alignment of the partial VP1/2A junction region of environmental and clinical HAV strains. A point mutation that resulted in the amino acid change is indicated by an arrow. Dots indicate conserved nucleotides.

5.6 Conclusion

This chapter presents the first genotyping information on clinical norovirus isolates during gastroenteritis outbreaks and selected waterborne viral pathogens circulating in water and wastewater environments in Singapore. Human enteric viruses with great genetic diversity were detected in both wastewaters and surface waters, indicating the presence of human origin of fecal contamination in highly urbanized water catchment areas. Rapid and accurate identification of sources of fecal contamination has important implications for protecting catchment water quality.

The present study demonstrates a genetic diversity of both noroviruses GI and GII in various environmental water samples. This is in contrast to the situation measured in the human population in Singapore during the same period, particularly norovirus-associated gastroenteritis outbreaks, when single norovirus strain usually dominated. The circulation of multiple genotypes of enteroviruses and astroviruses in water environments was also observed during the study period. It is surprising that a small region like Singapore with total area of 710.2 km² and a population of 4.99 million, contained a great genetic diversity of enteric viruses in urban water catchments and wastewater samples. This may indicate that multiple genotypes of these viral pathogens circulate concurrently in the same community. The present study provides evidence about the possible introduction of these viruses from individuals traveling between different geographical regions.

The present study, based on direct molecular characterization of environmental isolates of enteric viruses, improves our understanding of the epidemiology and the possible role of water as a vehicle for transmission of the emerging noroviruses and other important waterborne enteric viruses in this tropical region. The procedures applied here for genotyping enteric viruses in the community sewage and surface waters should be useful tools for studying long-term changes in epidemiological patterns and environmental dynamics of selected viral pathogens.

Chapter 6

Conclusions and Recommendations

Human enteric viruses have emerged as important causes of major waterborne disease outbreaks in recent years worldwide. The occurrence of enteric viruses in aquatic environments constitutes a major health hazard because of their low infective dose and resistance to environmental breakdown. Currently, our knowledge on the occurrence of enteric viruses in tropical water environments is limited. This thesis describes the prevalence and molecular characterization of enteric viruses in urban water catchments and community wastewaters in Singapore. In this chapter, results of the present study are summarized and future research issues in this field are discussed.

6.1 Conclusions

As discussed in Chapter 2, the detection of enteric viruses in environmental waters constitutes special challenges and multi-stage concentration procedures of water samples are needed. In the present study, tangential flow filtration has proven useful for the simultaneous concentration of multiple enteric viruses of concern in environmental water samples. The concentration processes of viruses as described in Chapter 3 using this emerging ultrafiltration method may be applied to the routine monitoring of virological quality of water sources. Molecular methods were used for the direct detection of human enteric viruses in concentrated environmental water samples due to their advantages of high speed, specificity and sensitivity. The advent of molecular methods has allowed the detection of enteric viruses, particularly noroviruses previously undetected due to their inability to grow in conventional cell culture.

Sewage has been recognized as an important source of waterborne pathogens in urban watersheds. A baseline understanding of diverse groups of enteric viruses that occur in wastewaters in Singapore was determined in order to determine which viruses

are effective indicators of fecal contamination. In addition, monitoring of viral pathogens in urban wastewater indicates the infections circulating in a given population and the integrity of the sewer system. All five tested human enteric viruses (adenoviruses, astroviruses, enteroviruses, hepatitis A viruses and noroviruses) were detected in wastewater samples collected from all water reclamation plants. Adenoviruses, astroviruses and both noroviruses GI and GII were found in 100% of the sewage and secondary effluent samples. Average concentrations of human enteric viruses in urban sewage and secondary effluent were 10^4 to 10^6 gene copies/100ml and 10^3 gene copies/100ml, respectively, as determined by QPCR.

The occurrence of enteric viruses in Marina Bay, the most highly urbanized water catchment areas of Singapore, was studied from June 2006 to January 2009. Noroviruses were the most prevalent enteric virus groups detected in the present study, followed by astroviruses, enteroviruses, adenoviruses and hepatitis A viruses. Adenoviruses and noroviruses GII were also quantified by QPCR, with concentrations ranging from 10^2 to 10^3 gene copies/liter and 10^3 to 10^4 gene copies/liter, respectively in urban rivers and canals. Results of the viral occurrence study indicate that the rivers and canals in Marina Bay areas are being impacted by human fecal material carrying human enteric viruses. Further comprehensive surveillance of human enteric viruses should be conducted to evaluate the performance of the current ongoing sewer rehabilitation programme along the waterways. No significant correlation was found between the levels of bacterial indicator and the presence of enteric viruses. This suggests that monitoring of microbiological quality of source waters by using only bacterial indicators is not sufficient for assessment of the presence of pathogenic viruses.

Molecular characterization of virus-positive samples was performed to provide an understanding of the spread of individual viral strains isolated from water environments in Singapore. Molecular cloning and sequence analysis revealed the genetic diversity of astroviruses, enteroviruses and noroviruses. The circulation of several viral strains identical to, or closely related to, other strains isolated previously in highly divergent geographical regions worldwide was observed. This suggests that

globalization accelerates the pace of movement of goods and people around the world and may thus increase risks of the spread of infectious diseases by transporting waterborne pathogens.

Norovirus isolates from environmental samples were compared to clinical isolates in Singapore during gastroenteritis outbreaks. Genotyping of clinical isolates collected during the same period as the environmental surveys clustered all norovirus isolates to GII/4. In contrast, genotyping of environmental norovirus isolates showed a wide spectrum of genotypes of GI and GII, in addition to the GII/4 strains. Results of the present study clearly indicate that noroviruses detected in both clinical and environmental samples are closely related. This suggests that fecal contaminated surface water used for recreational activities could pose a potential risk of norovirus infection. The great diversity of both norovirus GI and GII suggests the rapid emergence and circulation of these viral pathogens within and beyond a given geographical area. Unclassified norovirus genotypes of GI and GII were identified and they could be novel norovirus strains that emerged particularly in the Asian region. It is expected that more novel and recombinant genotypes of norovirus are likely to be discovered in the future. Noroviruses have been identified as etiologic agents of acute gastroenteritis across all age groups worldwide. From a global perspective, the present study improves our understanding of the ecology of noroviruses as various norovirus genotypes may be circulating globally.

An unusual distribution of enteroviruses in environmental samples was observed, with coxsackieviruses A of HEV-C being the most predominant enterovirus strains in Singapore. This group of human enterovirus has rarely been detected both in clinical and environmental samples as they grow poorly in conventional cell culture. The present study highlights the importance of molecular techniques for the detection of difficult-to-cultivate enteric viral strains. A newly discovered HEV-A strain, enterovirus 89, was also detected in both sewage and surface water samples. The identification of human rhinovirus in environmental waters suggests a need for further study of this respiratory virus in recreational waters. Genotyping of environmental astrovirus isolates revealed the prevalence of HAstV-1. In addition, uncommon

(HAstV-2 and HAstV-4) and rare (HAstV-6 and HAstV-8) astrovirus genotypes were also identified. In contrast, there was less genetic diversity in environmental isolates of adenoviruses as compared to RNA viruses. The majority of adenovirus isolates in the present study were identified as enteric adenovirus type 41.

The results of the present study suggest that noroviruses GI and GII, which are mostly human pathogens as confirmed by DNA sequencing, have potential as viral indicators of fecal contamination in surface water since they were prevalent in water catchments and were detected in 100% of sewage samples in Singapore. The RT-PCR detection of noroviruses may be useful as an index of the presence of human pathogenic viruses in water environments. Moreover, noroviruses have been identified as etiological agents that caused several recent gastroenteritis outbreaks in Singapore (MOH 2006; MOH 2007; MOH 2009). Further comprehensive field study is needed to obtain a long-term quantitative data on their occurrence in water catchments of Marina Bay and other catchment areas in Singapore. The high prevalence of adenovirus type 41 in sewage also suggests that this pathogen could be used as an indicator for evaluating the viral inactivation efficiencies of advanced treatment processes in water reclamation plants. Furthermore, adenovirus type 41 has been shown to be more resistant to UV irradiation than other adenovirus serotypes (Ko et al., 2005; Baxter et al., 2007).

The present study provides valuable first data on the occurrence and molecular characterization of human enteric viruses in potential water sources for drinking and recreational activities in Singapore. This baseline understanding of human pathogenic viruses in water environments will enable microbial risk assessment in urban water catchments to be made and contamination prevention strategies to be addressed. Results of viral occurrence in urban wastewaters indicate that wastewater surveillance by molecular techniques could be a useful tool for rapid monitoring and characterizing various groups of viral pathogen circulating in the community in order to complement the conventional epidemiological investigation and reporting practices. The global outbreaks of severe acute respiratory syndrome (SARS) and avian influenza and the recent increase in norovirus activity in Singapore highlight the vulnerability of high-

density urban communities to pathogenic viruses. Direct surveillance for potential waterborne viral pathogens in drinking and recreational water sources may thus be warranted to better protect public health.

6.2 Recommendations

The high prevalence of human enteric viruses in Marina catchment areas have prompted further study to examine the presence of infectious enteric viruses in source waters for drinking and recreational purposes in Singapore by using integrated cell culture-PCR assays. Culture of wild-type virus in environmental samples requires a larger quantity of virus or requires many months of serial cell culture passage to grow the low quantity of virus present in environmental water samples. Use of the integrated cell culture-PCR will provide a rapid and accurate method for enumerating infectious viruses in environmental samples (Reynolds et al., 1996). Moreover, the combination of both methodologies could be used to detect infectious hepatitis A virus in environmental water samples which is usually not cytopathic in infected cells (Jiang et al., 2004). Better knowledge of the infectivity of human enteric viruses recovered from environmental water samples would improve estimates of the overall pathogen risk for public health.

A great genetic diversity of enteric viruses in the present study suggests the need for analytical tools such as microarrays for the rapid and high throughput detection and characterization of multiple viruses in various environmental samples. An interesting approach using a long oligonucleotide (70-mer) DNA microarray for simultaneously detecting hundreds of animal and human viruses has been proposed (Wang et al., 2002). The authors demonstrated that this approach coupled with a random PCR amplification strategy, was able to detect multiple viruses and discriminate among viral subtypes. A recent proof of concept study by Brinkman and Fout (2009) demonstrated that microarrays could be used to genotype identify different noroviruses seeded in environmental water samples. Further study is needed to evaluate this method for the rapid genotyping of different viruses in real environmental water samples.

The viral concentration quantified by QPCR in the present study represents a conservative estimation of enteric viruses in the water environment because the final figure was not corrected by the probable loss during nucleic acid extraction and reverse transcription step for RNA viruses. It has been suggested that controls are needed to evaluate the efficiency of nucleic acid extraction steps and reverse transcription for estimating viral concentration by QPCR (Bosch et al., 2008). The use of mengovirus which belongs to the *Picornaviridae* family, has been proposed for controlling the steps of RNA extraction (Costafreda et al., 2006). For reverse transcription, single-stranded RNA molecules which are similar to the viral target have been developed for use as internal controls in RT-PCR assays (Parshionikar et al., 2004; Costafreda et al., 2006). These controls should be used in the future study for an accurate quantification of viral gene copies by QPCR in various environmental samples.

Little is known about the fate of human enteric viruses in the tropical aquatic environment, since most studies have been conducted in temperate and cold regions. Future research is recommended to provide quantitative data on the survival of specific enteric viruses in the tropical water environment under different environmental stresses such as sunlight irradiation, salinity and sediments. Such data could be used to develop prediction models for predicting the fate and transport of viral pathogens in surface waters. It has been shown that sunlight inactivation increases with increasing salinity for indicator bacteria and bacteriophages (Sinton et al., 2002). This is relevant to Marina Bay as its conversion to a freshwater reservoir will result in decreasing salinity and possibly a reduced effect of sunlight inactivation for the target viruses. It has also been shown that radicals (such as singlet oxygen) generated by sunlight interacting with external sensitizer molecules may play an important role in the photoinactivation of coliphages in water (Kohn and Nelson 2007). The role of different reactive oxygen species formed by sunlight in inactivating selected human enteric viruses in surface water should be investigated. Sediments also play an important role in the removal or inactivation of viruses either by sorption or indirectly through affecting the sunlight irradiation in the water column (Templeton et al., 2008). More recently, Skrabber et al. (2009) demonstrated that bacteriophages were more frequently detected in freshwater

sediments than the overlying water, suggesting further study is needed on the fate of entrapped pathogenic viruses in sediments.

In the case of viral inactivation during water and wastewater treatment processes, changes in disinfection techniques to reduce the production of carcinogenic disinfection by-products can change the relative importance of waterborne viruses. For example, using UV light to disinfect water and wastewater to eliminate the production of chloramines can make it difficult to control adenoviruses, which are the most resistant waterborne pathogen to UV light inactivation. Currently, UV disinfection is used as a post-operative procedure for reverse osmosis in the production of reclaimed water in Singapore. The prevalence of enteric adenoviruses in water and wastewater environments in the present study suggests the need for more comprehensive field investigation to examine the response of these viral pathogens to UV disinfection.

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APPENDIX

Distribution of enteric viruses in surface water samples collected from urban water catchments as determined by nested PCR/RT-PCR^a

Sampling date	Sampling location	Adenovirus	Astrovirus	Enterovirus	Norovirus GI	Norovirus GII	Hepatitis A virus
22-Jun-2006	Singapore River (Downstream)	+	-	-	-	+	NT
	Kallang River						
	Upstream	-	-	-	+	-	NT
	Downstream	-	-	-	+	-	NT
	Stamford Canal	+	+	+	+	+	NT
4-Jul-2006	Singapore River (Upstream)	-	-	-	-	-	NT
	Kallang river (Upstream)	-	-	-	-	-	NT
	Stamford Canal	-	+	+	-	+	NT
11-Jul-2006	Singapore River (Upstream)	-	-	-	-	-	NT
	Kallang river (Upstream)	-	-	-	-	-	NT
	Stamford Canal	-	+	-	-	-	NT

APPENDIX (continued)

Sampling date	Sampling location	Adenovirus	Astrovirus	Enterovirus	Norovirus GI	Norovirus GII	Hepatitis A virus
18-Jul-2006	Singapore River (Downstream)	+	-	-	-	-	NT
	Kallang River						
	Upstream	-	-	-	-	-	NT
	Downstream	-	-	-	-	-	NT
	Stamford canal	+	-	+	-	+	NT
24-Jul-2006	Singapore River (Downstream)	-	-	+	-	-	NT
	Kallang River						
	Upstream	-	-	-	-	+	NT
	Downstream	-	-	-	+	+	NT
	Stamford canal	-	+	-	+	+	NT
15-Aug-2006	Singapore River						
	Upstream	-	-	-	-	+	NT
	Downstream	-	-	-	-	+	NT
	Kallang River						
	Upstream	+	+	+	+	+	NT
	Downstream	-	-	-	+	+	NT
	Stamford canal	-	+	+	+	+	NT

APPENDIX (continued)

Sampling date	Sampling location	Adenovirus	Astrovirus	Enterovirus	Norovirus GI	Norovirus GII	Hepatitis A virus
3-Oct-2006	Singapore River						
	Upstream	+	+	-	+	+	-
	Downstream	+	+	-	-	-	-
	Kallang River						
	Upstream	+	+	-	+	+	-
	Downstream	+	+	-	-	+	-
	Stamford canal	-	-	-	-	-	-
25-Oct-2006	Geylang River						
	Upstream	+	+	+	+	+	NT
	Downstream	-	+	-	+	+	NT
1-Nov-2006	Singapore River						
	Upstream	+	-	+	-	+	-
	Downstream	-	-	-	+	-	-
	Kallang River						
	Upstream	+	+	-	-	+	-
	Downstream	+	+	+	+	+	-
	Stamford canal	+	+	+	+	+	-

APPENDIX (continued)

Sampling date	Sampling location	Adenovirus	Astrovirus	Enterovirus	Norovirus GI	Norovirus GII	Hepatitis A virus
20-Nov-2006	Singapore River						
	Upstream	+	+	+	-	+	-
	Downstream	+	+	+	+	+	-
	Kallang River						
	Upstream	+	+	+	+	+	-
	Downstream	+	+	+	-	+	-
	Stamford canal	+	+	+	+	+	-
11-Dec-2006	Singapore River						
	Upstream	-	+	-	+	+	-
	Downstream	+	+	-	+	+	-
	Kallang River						
	Upstream	+	+	-	+	+	-
	Downstream	-	+	-	+	+	-
	Stamford canal	+	+	+	+	+	+

APPENDIX (continued)

Sampling date	Sampling location	Adenovirus	Astrovirus	Enterovirus	Norovirus GI	Norovirus GII	Hepatitis A virus
27-Dec-2006	Singapore River						
	Upstream	-	-	-	-	-	-
	Downstream	-	-	-	-	+	-
	Kallang River						
	Upstream	+	+	-	-	+	-
	Downstream	-	+	-	-	-	-
	Stamford canal	+	+	+	+	+	+
	Marina Bay						
14-Mar-2007	Station 1	+	+	+	+	+	NT
11-Apr-2007	Station 2	-	+	+	+	-	NT
9-May-2007	Station 3	-	-	-	-	+	NT
4-Jul-2007	Station 4	-	-	-	-	-	NT
4-Jul-2007	Station 5	-	-	-	-	-	NT
4-Jul-2007	Station 6	-	-	-	-	-	NT
4-Apr-2007	Bukit Timah canal	+	-	+	-	+	NT
	Alexandra canal	-	-	+	-	-	NT

APPENDIX (continued)

Sampling date	Sampling location	Adenovirus	Astrovirus	Enterovirus	Norovirus GI	Norovirus GII	Hepatitis A virus
23-May-2007	Rochor Canal	-	-	-	+	+	NT
20-Jun-2007	Geylang River (upstream)	-	-	+	-	+	NT
11-Dec-2007	Kallang River						
	Upstream	-	+	NT	+	+	NT
	Downstream	-	+	NT	+	+	NT
	Stamford canal	-	-	NT	+	+	NT
	Rochor canal	-	-	-	-	-	NT
14-Feb-2008	Kallang River						
	Upstream	-	NT	+	+	+	-
	Downstream	+	NT	+	+	+	-
	Stamford canal	-	NT	+	+	+	-
	Rochor canal	-	NT	NT	+	+	-

APPENDIX (continued)

Sampling date	Sampling location	Adenovirus	Astrovirus	Enterovirus	Norovirus GI	Norovirus GII	Hepatitis A virus
17-Oct-2008	Kallang River (Upstream)	+	+	-	+	+	-
	Geylang River	-	+	-	+	+	-
	Stamford canal	+	+	+	+	+	-
	Rochor canal	-	-	+	+	+	-
3-Nov-2008	Kallang River (Upstream)	-	-	-	-	-	-
	Geylang River	-	-	-	+	-	-
	Stamford canal	-	+	+	+	+	-
	Rochor canal	-	+	+	+	+	-
26-Dec-2008	Kallang River (Upstream)	-	-	-	+	-	-
	Geylang River	-	-	-	-	-	-
	Stamford canal	-	+	+	+	+	+
	Rochor canal	+	-	+	+	+	-
20-Jan-2009	Kallang River (Upstream)	-	-	-	+	+	-
	Geylang River	-	-	-	-	+	-
	Stamford canal	-	+	+	+	+	+
	Rochor canal	-	+	-	-	+	-

^a +, viruses were detected; -, viruses were not detected; NT, non-tested.