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ROTAVIRUS IN DRINKING WATER – MOLECULAR METHODS FOR MEASUREMENT OF INFECTIVITY

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Tiivistelmä — Referat — Abstract

Quantitative reverse transcription PCR (RT-qPCR) assay is widely used for the detection of RNA viruses in environmental water samples. However, a major limitation of using RT-qPCR assay to quantify virus titers is its inability to discriminate between infectious and non-infectious viruses, resulting in overestimation of viral infectivity. Thus, the aim of this study was to develop a reliable molecular method for rotavirus detection with information on viral infectivity, and which may contribute to the development of molecular detection methods for correct estimation of infectivity of non-cultivable viruses.

In experimental work, the potential of using propidium monoazide (PMA) or RNase treatment prior to RT-qPCR assay was evaluated to measure the infectivity of human rotavirus. In brief, original human rotavirus (HRV) stock was produced by propagating viruses in MA-104 cells. The virus stocks (including HRV stock A and B) were thermally treated at 80 °C at different time points. The virus titer was measured by (1) cell culture-based infectivity assay, (2) RT-qPCR assay, and (3) RT-qPCR assay with PMA or RNase pretreatment. The result of cell culture-based infectivity assay showed that heat exposure for 5 min at 80 °C was sufficient to inactivate the HRV, while RT-qPCR assay alone overestimated the viral infectivity. The results of RT-qPCR assay with pre-treatments showed that, for thermally-inactivated HRV stock A, similar level of false-positive results was reduced with PMA treatment regardless of inactivation time (ranges from 1.04 to 1.18 $\log_{10}$ PCR-units), while higher reduction level was observed with RNase treatment (ranges from 2.64 to 2.89 $\log_{10}$ PCR-units). On the other hand, the effects of both pre-treatments on thermally-inactivated HRV stock B were negligible.

In conclusion, both PMA and RNase pre-treatments eliminated the false-positive results of RT-qPCR assay to some extent in defined conditions, while the discrepancy between the infectivity assay and RT-qPCR assay even with PMA or RNase treatment was observed. In order to confirm the potential of using RT-qPCR assay combined with pre-treatments to measure the infectivity of rotavirus, further studies on optimization of PMA and RNase treatments and production of optimal virus stock would be necessary.

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Rotavirus, RT-qPCR, PMA, RNase, Viral infectivity

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PREFACE

This study was carried out from September 2013 to May 2014 in the Department of Food Hygiene and Environmental Health in the Faculty of Veterinary Medicine. This study was financially supported by European Union, as a part of research project – the 'Aquavalens' project number 311846 which aims to improve the safety of European drinking water through developing more rapid methods of detecting viruses, bacteria and parasites in water.

I would like to thank my supervisor Dr. Leena Maunula for giving me a chance to work in her research group and her support. I want to extend my gratitude to PhD student Satu Oristo for her support and her guidance on experimental research, and also to research assistant Kirsi Söderberg for her practical advices during experimental research.

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ABBREVIATIONS

ATCC	American Type Culture Collection
CDC	Centers for Disease Control and Prevention
CPE	Cytopathogenic effect
Cq	Quantification cycle
EMA	Ethidium Monoazide
HRV	Human Rotavirus
ICC-PCR	Integrated Cell Culture-Polymerase Chain Reaction
IMS-PCR	Immunoseperation-Polymerase Chain Reaction
NSP	Non-structural protein
PCR	Polymerase Chain Reaction
PMA	Propidium Monoazide
qPCR	Quantitative Polymerase Chain Reaction
RCWG	Rotavirus Classification Working Group
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RT-qPCR	Reverse Transcriptase-Quantitative Polymerase Chain Reaction
$TCID_{50}$	50% tissue culture infectious dose
VP	Viral protein
WHO	World Health Organization

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1. INTRODUCTION

Human rotavirus is a leading cause of viral gastroenteritis in infants and children worldwide, and it is responsible for 37% of all diarrhea deaths in children under five and 50-60% of acute gastroenteritis cases of hospitalized children throughout the world (WHO 2011; Tate and others 2012). WHO estimates that approximately 453 000 children aged under 5 years died during 2008 due to rotavirus infection; the vast majority (95%) of these children live in developing countries with poor hygienic situations (WHO 2012). On the other hand, the deaths from rotavirus infection in developed countries are rare, but it remains the one of most common causes of hospitalization for acute gastroenteritis in children (Leung and others 2005; Widdowson and others 2005).

It is generally known that the transmission of rotavirus mainly occurs by faecal-oral routes. Humans become infected by person-to-person contact and the inhalation of airborne human rotaviruses as well as the ingestion of water or food contaminated with human rotaviruses (WHO 2011b). Although the ingestion of drinking water is not the most common way of exposure, the presence of human rotaviruses in drinking water poses a public health risk due to high morbidity rates at low infectious doses (WHO 2011b). Waterborne transmission may be facilitated due to the stability of rotavirus in environmental water and its resistance to disinfection treatments (He and others 2009). Occasional waterborne outbreaks have been caused by consumption of drinking water contaminated with human rotaviruses (Hopkins and others 1984; Villena and others 2003; Koroglu and others 2011; Mellou and others 2014).

Accordingly, the availability of a reliable and reproducible method for detection of rotavirus in environmental samples is crucial to identify the infectious risk for public health and to reduce their impact on public health (Rosa and Muscillo 2013). However, the virological analysis of environmental water samples has been historically challenging mainly due to the low concentration of target viruses as well as the presence of inhibitors in environmental water (Hamza and others 2011; Rosa and others 2012; Gensberge and Kostic 2013). Particularly, the environmental analysis of rotavirus has more difficulties and requires different methodological protocols than other enteric viruses (Ruggeri and Fiore 2013).

Basically cell culture is the gold standard method to examine the infectivity of rotavirus (Hamza and others 2011). However, it is not sufficiently sensitive for all rotavirus strains, and it is difficult to perform and time consuming (Ruggeri and Fiore 2013). Currently, the detection of viral genome using PCR-based molecular assays is the only way to identify the infectious risk for the population (Gassilloud and others 2003). Especially, quantitative PCR assays (qPCR or RT-qPCR) have become the method of choice for the detection and quantitation of health-significance viruses including rotavirus, and this approach is widely used in the field of food and environmental virology and continuously evolving (Yeh and others 2009; Bosch and others 2011; Rodriguez-Lazaro and others 2012). However, one major limitation of using these assays is that they detect and quantify both infectious and non-infectious viral genomes, and in consequence they do not provide correct information of viral infectivity (Fittipaldi and others 2011). Indeed, numerous studies have showed that PCR-based assays resulted in overestimation of viral infectivity (Gassilloud and others 2003; Duizer and others 2004; Bosch and others 2008, 2011).

In order to overcome the limitations of current methodologies, some promising methods to measure the viral infectivity has been proposed. Among several approaches, pre-treatments of viral sample using dyes (EMA and PMA) or enzymes (Proteinase K and RNase) combined with qPCR assays would appear to be relatively easy and rapid to perform. In addition, the applicability of both pre-treatments to certain human viruses has found to be successful in some cases to discriminate infectious and inactivated viruses. However, the applicability of such assays to measure the infectivity of rotavirus has not been thoroughly investigated yet.

The present study aimed to develop a reliable and rapid molecular method to quantify the infectivity of human rotavirus as health-significance virus in drinking water, and further to contribute to the development of molecular methods for correct estimation of infectivity of non-cultivable health-significant viruses such as human norovirus.

In the first part of the thesis, the general aspects of rotavirus and rotavirus disease, the significance of rotavirus in drinking water, as well as current and promising methods for rotavirus detection in environmental water samples are reviewed. In the second part, the potential of using RT-qPCR assay combined with enzymatic treatment or dye treatment to assess the infectivity of human rotavirus are investigated and evaluated.

2. LITERATURE REVIEW

2.1 Rotavirus

2.1.1 Historical background

In 1973, Bishop and colleagues discovered new virus particles in the epithelial cells of duodenal mucosa from children with acute gastroenteritis (Bishop and others 1973). The viruses with similar morphological appearance had been found in the intestinal epithelium of infant mice with diarrhea in 1963 (Adams and others 1963). A year after Bishops' discovery in 1974, Flewett and colleagues named rotavirus after the Latin word *rota* (=wheel) plus virus, since the shape of virus particles resembled the spokes of wheel under electron microscopy as described in Figure 1 (Flewett and others 1974).

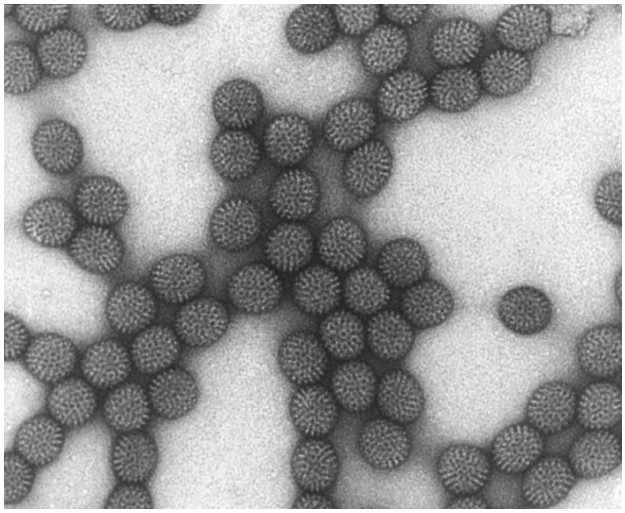

Figure 1. Wheel-like shape of rotaviruses under electron microscopy. Adapted from CDC 2011.

2.1.2 Structure and genome

Rotaviruses are the members of the *Reoviridae* family (Matthews and Maurin 1979). The virion of rotaviruses is characterized as a 70-nm non-enveloped icosahedral particle with a capsid (Wilhelmi and others 2003). As shown in Figure 2A, the virion possesses a viral genome consisting of 11 segments of double-stranded RNA (dsRNA), and each RNA segment encodes one protein, except the segment 11 which encodes two proteins (Greenberg and Estes 2009). There are 12 proteins encoded: 6 structural viral proteins (VP1, VP2, VP3, VP4, VP6 (VP5+VP6), and VP7) and 6 non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6), and each of which plays a different role during virus life cycle; including cell entry, transcription and replication (Jayaram and others 2004; Hu and others 2012).

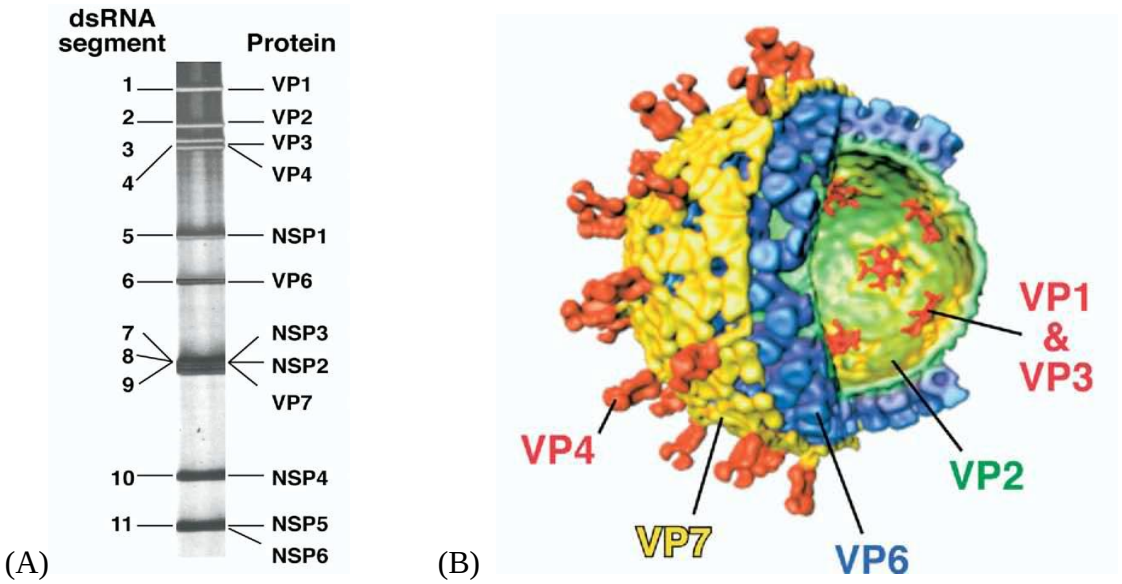

Figure 2. Structure and proteins of rotavirus. **(A)** The viral genome of 11 double-stranded RNA segments is analyzed by polyacrylamide gel electrophoresis, and each of 11 gene segments encodes one protein except the segment 11, and thus there are 12 proteins encoded: 6 structural viral proteins (VP1, VP2, VP3, VP4, VP6 (VP5+VP6), and VP7) and 6 non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6). **(B)** The structure of rotavirus virion is determined by image reconstruction after electron cryomicroscopy, and viral proteins (VP) construct three concentric protein layers of rotavirus capsid and the capsid of rotavirus is structured to protect its genome. Adapted from Greenberg and Estes 2009.

The capsid of rotavirus is structured to protect its genome and deliver it successfully into a suitable host cell, in which the genome is replicated and the virus particles make copies (Jayaram and others 2004). As depicted in Figure 2B, viral proteins (VP) construct three concentric protein layers of rotavirus capsid, and they include an outermost layer, an intermediate layer and an inner layer (Jayaram and others 2004). The outermost layers are implicated in cell attachment, membrane penetration and cell entry (Patton 2013). They are mostly composed of glycoprotein VP7 and the spikes of protease-activated attachment protein VP4 (Patton 2013). VP7 is a Ca^{2+} binding protein and a key mediator of Ca^{2+} driven uncoating of the outermost layer, initiating the replication cycle (Trask and others 2012). VP4 is susceptible to proteolysis; e.g. trypsin, so that it is cleaved into VP8 and VP5, enhancing viral infectivity by several folds and facilitating virus entry into cells (Clark and others 1981; Carter and Saunders 2007). The sole component of the intermediate layer is VP6, and it surrounds the inner layer (Leung and others 2005). VP6 maintains structural integrity during the process of endogenous transcription, and provides mRNA exit channels (Jayaram and others 2004). The inner layer of the virion is composed of the core shell protein VP2. The viral RNA-dependent RNA-polymerase VP1 and capping enzyme VP3 are attached to the inside of the VP2 layer (Patton 2013). Together VP1, VP2 and VP3 represent the core of rotavirus virion (Leung and others 2005).

The ds RNA genome encodes its own enzymes necessary for transcription and it is capable of endogenously transcribing its genome (Jayaram and others 2004). The capped (+) RNA transcripts encode the viral proteins and function as templates for the production of (-) RNA to make the progeny ds RNA (Jayaram and others 2004). The mode of replication is conservative since the ds RNA of the infecting virion remains intact (Carter and others 2007). Once enough viral proteins have been made in cells, the RNA genome is replicated and packaged into newly made double-layer particle in specialized structures called viroplasms (Jayaram and others 2004). At the final stage of replication, virus assembly takes place by the addition of outer layer of the capsid and the spikes, and then virions are released from the cells either by lysis or by exocytosis (Carter and others 2007).

2.1.3 Classification

Rotaviruses have been classified into five serological groups A to E and two tentative groups F and G, mainly on the basis of antigenic specificity of VP6, or more recently sequence analysis of VP6 (Kindler and others 2013). Rotaviruses belonging to group A, B and C are known to induce infections in both humans and animals, whereas group D, E, F and G are only in animals (Matthijnssens and others 2010). Additionally, another group of rotaviruses originally named ‘new adult diarrhea viruses or ADRV-N was discovered, and recently renamed as group H (Matthijnssens and others 2010; Kindler and others 2013).

Group A rotaviruses are the leading causes of viral diarrhea, accounting for nearly all rotavirus-associated mortality and morbidity, especially in children less than 5 years of age (Patton 2013). In consequence, group A rotaviruses have been extensively studied and have been classified further using various approaches (Table 1). Currently available vaccines are directed against the common group A human rotaviruses (Matthijnssens and others 2010; Ghosh and Kobayashi 2011). Group B rotaviruses are genetically and antigenicallly distinct from group A rotaviruses, and they causes severe, cholera-like diarrhea mostly in adults (Yamamoto and others 2010). They were first identified as adult diarrhea rotaviruses (ADRV), which have caused large outbreaks of severe diarrhea involving thousands of adults in China (Hoshino and Kapikian 2000). It has been only detected in China, India, Bangladesh, and recently in Myanmar (Yamamoto and others 2010). Group C rotaviruses tend to cause sporadic outbreaks and they have been occasionally associated with food-borne contamination (Patton 2013). Group D and E rotaviruses are known to infect avian species (Patton 2013). Group F and G rotaviruses have been originally identified in chicken in 1984, and have been only found in birds so far

(Kindler and others 2013). Group H rotaviruses (also called ADRV-N) have been only isolated in a large outbreak among adults in China, with a sporadic case in Bangladesh (Matthijnssens and others 2010).

Table 1. Classification of group A human rotaviruses. Group A rotaviruses have been categorized based on (i) whole-genome RNA hybridization patterns (genogroups); (ii) the antigenic properties of VP6, VP7 and VP4 (subgroups, G-serotypes and P-serotypes, respectively); (iii) the nucleotide sequence analysis of VP7 and VP4 (G-genotypes and P-genotypes); and (iv) the migration pattern of the RNA genome segments when subjected to polyacrylamide gel electrophoresis (long, short, supershort or atypical electropherotypes). Adapted from Maunula 2001.

Classification	Types	Based on identification of
Within Group A		
Genogroup	WA-like, DS-1-like, and AU-1-like	RNA-RNA hybridization
Subgroup	I, II	Antigenic specificities of VP6
G-serotypes (G:glycoprotein) or G-genotypes	G1, G2…etc	Antigenic properties of VP7 or sequence analysis of VP7
P-serotypes (P:proteinase)	P1, P2…etc	Antigenic properties of VP4
P-genotypes	P[1], P[2] etc	Sequence analysis of VP4
Electropherotypes	long, short, supershort or atypical types (e-types)	Patterns of 11 genes after gel electrophoresis of genomic RNA

Recently, a whole genome-based genotyping scheme of group A rotaviruses, also known as the Rotavirus Classification Working Group (RCWG) genotyping system has proposed (Matthijnssens and others 2008). This RCWG system has recommended to use a uniform nomenclature in defining the complete genotype constellation of group A rotaviruses, with notation Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (x representing the genotype number) being used to denote the genotype of VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5 genes respectively (Matthijnssens and others 2011). Until 2011, the whole genomes of at least 167 group A human rotaviruses, a limited number of group B and C have been analyzed (Ghosh and Kobayashi 2011). In addition, only one complete genome sequence exists for group D and two genome sequences are available for group H, but no sequence available for group E yet (Ghosh and Kobayashi 2011). Recently, first complete genome sequences of group F and G have been described (Kindler and others 2013).

2.1.4 Diversity and Evolution

Human rotaviruses display a considerable genetic diversity (Ghosh and Kobayashi 2011). Among different groups A, B and C, the sequence identity of 11 gene segments is generally less than 60 % (Alam and others 2007; Nagashima and others 2008). The high level of genetic diversity within a group and different level of sequence diversity in each 11 gene segment have been observed (Matthijnssens and others 2008). Moreover, there are at least four mechanisms which generate and increase the overall diversity of rotaviruses and by which rotaviruses evolve: (i) point mutation or "drift", (ii) genomic reassortment or "shift", (iii) gene rearrangement, and (iv) interspecies transmission (Iturriza-Gomara and others 2001; Matthijnssens and others 2009; Ghosh and Kobayashi 2011).

The accumulation of point mutations is believed to occur frequently in human rotaviruses due to the error-prone nature of viral RNA-dependent polymerase (Matthijnssens and others 2009). It results in changes in the gene sequence and thus may affect the function of the viral proteins (Ghosh and Kobayashi 2011). Genomic reassortment is an exchange and substitution of RNA segments between different rotavirus strains (Ghosh and Kobayashi 2011). Due to the segmented nature of rotavirus genome, it is a major evolutionary mechanism in commonly circulating rotavirus strains (Matthijnssens and others 2009; McDonald and others 2009). Most reassortment events across genogroups occurs in the genes encoding VP4 and VP7, resulting in the formation of new strains with unusual G/P combinations and contributing to overall diversity (Iturriza-Gomara and others 2001; Cunliffe and others 2002).

In addition, gene arrangement within single RNA segment, such as deletions, duplications and insertions, causes a change in the size of the RNA segment. However, the overall contribution of gene arrangement to the diversity of rotaviruses seems to be lower than other mechanisms (Matthijnssens and others 2009). Moreover, the interspecies transmission between animal and human rotaviruses is known to be another major mechanism generating the diversity of human rotaviruses (Gentsch and others 2005). Previous studies have detected several human rotaviruses which have close relations to animal strains; for example, feline-like rotaviruses in children in Japan (Nakagomi and Nakagomi 1989), porcine serotype G5 rotavirus in Brazilian children (Gouvea and others 1994), and bovine-like serotype G8 in children in Malawi (Cunliffe and others 2001).

2.2 Rotavirus disease

2.2.1 Burden of rotavirus disease

Nearly all children by 5 years of age have been infected by rotavirus at least once, in both developed and developing countries (WHO 2013). It accounts for 37% of all diarrhea deaths in children under five and 50-60% of acute gastroenteritis cases of hospitalized children throughout the world (WHO 2011; Tate and others 2012). The rotavirus infections result in estimated 25 million outpatient visits and 2 million hospitalizations each year worldwide (Parashar and others 2003, 2009). WHO estimated that globally around 453 000 (420 000 - 494 000) child deaths occurred during 2008 due to rotavirus infections, and most deaths (95%) occurred in malnourished infants living in socioeconomically disadvantaged rural regions in low-income countries, where access to healthcare is poor, particularly located in sub-Saharan Africa and Southeast Asia as shown in Figure 3 (WHO 2012). On the other hand, the deaths from rotavirus infections are rare in developed countries, in Australia, East Asia, Europe and North America, but the incidence of disease in young children is similar to that of developing countries (Chen and others 2012). Soriano-Gabarro and others (2006) estimated that rotavirus accounted for 231 deaths, 87,000 hospitalizations, almost 700,000 outpatient visits as well as 3.6 million episodes of rotavirus disease among the 23.6 million children less than 5 years of age each year from 2000 to 2003 in the European Union. Thus, in developed countries, rotavirus infection remains the one of most common causes of hospitalization for acute gastroenteritis in children and leads to major medical costs (Leung and others 2005; Widdowson and other 2005).

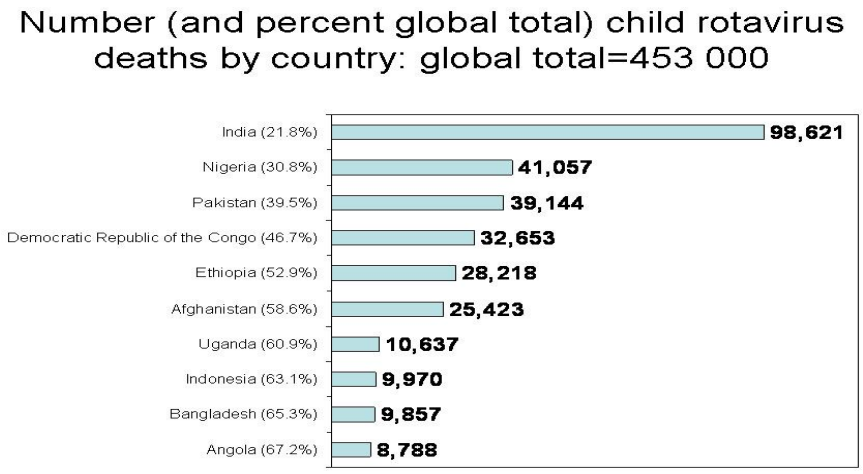

Figure 3. Estimated rotavirus deaths in 2008. National estimates of rotavirus attributable deaths among children under five years of age ranged from 98 621 (India) to fewer than 5 deaths (74 countries). Twenty-two percent of all rotavirus deaths under five years of age occurred in India. Five countries (India, Nigeria, the Democratic Republic of the Congo, Ethiopia and Pakistan) accounted for more than half of all rotavirus deaths under age five in 2008. Adapted from WHO 2012.

2.2.2 Clinical aspects of rotavirus disease

The primary site of rotavirus infection is mature enterocytes of the apical portion of villi in the small intestine causing gastroenteritis, and thus the main clinical manifestation is diarrhea (Greenberg and Estes 2009; Kindler and others 2013). Multifactorial and malabsorptive diarrheas may occur due to the virus-mediated destruction of absorptive enterocytes, the virus-induced suppression of absorptive enzymes, and functional changes in tight junctions between enterocytes leading to paracellular leakage (Greenberg and Estes 2009). In addition, the activation of enteric nervous system (ENS) and the effects of enterotoxin NSP4 (virus-encoded enterotoxin) seem to mediate secretory components of rotavirus diarrhea (Ramig 2004; Greenberg and Estes 2009). However, the mechanism of diarrhea induced by rotavirus is not fully understood (Ramig 2004; Greenberg and Estes 2009).

However, rotavirus infection is not always limited to the intestine (Greenberg and Estes 2009). Rotaviruses have been detected in the extra-intestinal sites of the liver, kidney, and central nervous system (CNS) (Ramig 2004; Dickey and others 2009; Greenberg and Estes 2009). Few cases include the finding of virus in the liver following fatal disease (Carlson and others 1978), the finding of elevated liver enzymes associated virus infection (Kitamoto and others 1993), and the demonstration of viral replication in the liver and kidneys of immune-deficient children (Gilger and others 1992). In addition, some clinical reports of rotavirus have shown that rotavirus spread and pathogenesis may play a potential role to cause viremia (Blutt and others 2003; Lynch and others 2003).

Rotavirus infections can result in asymptomatic infection, mild diarrhea or severe gastroenteritis, after the incubation period of 1 to 3 days (Bernstein 2009; Chen and others 2012). Rotaviral gastroenteritis is more severe than other causes of gastroenteritis, and it often results in dehydration, hospitalization and even death (Bernstein 2009). Although rotavirus disease can occur at any age, rotavirus gastroenteritis is most common and severe in children 3 to 36 months of age (Dennehy and others 2008; Chen and others 2012). The major symptoms in young children include mild-to-severe watery diarrhea, vomiting, and low-grade fever, and symptoms usually last for up to 4–8 days (Staat and others 2002; Lee and others 2008). Among young children, the first rotavirus infection is most likely to produce moderate-to-severe diarrhea disease, but the incidence of moderate-to-severe diarrhea decreases with second infections, and third infections are typically asymptomatic (Bernstein 2009).

Among adults, rotavirus infections have been associated with a wide spectrum of disease severity and manifestations; symptoms included diarrhea, fever, headache, malaise, nausea or cramping (Anderson and Weber 2004). Particularly, rotavirus infections in adults have been often related to epidemic outbreaks (Meurman and others 1977; Foster and others 1980; Griffin and others 2002) or traveler's diarrhea (Bolivar and others 1978; Vollet and others 1979; Steffen and others 1999). In addition, adults who are in contact with children are shown to be at particularly high risk of infection since transmission of rotavirus within families from children to parents seems to be a common event (Wenman and others 1979; Grimwood and others 1983).

Treatments primarily aim at symptom relief and the restoration of normal physiological functions, as rotavirus disease is usually self-limited (Anderson and Weber 2004). The rehydration and maintenance of proper fluid and electrolyte balance remains the mainstay of treatment (Leung and Robson 1989). WHO recommends the use of oral rehydration formula for children who are mildly to moderately dehydrated, whereas intravenous rehydration therapy is recommended for those who have severe diarrhea, intractable vomiting or for those too sick to take oral feedings (WHO 2006). In addition, resumption of a normal, age-appropriate diet is essential for maintaining the nutritional status of child, and it can reduce the morbidity and mortality of rotavirus gastroenteritis (Leung and Robson 1989; Nutrition committee 2003). The use of probiotics (*Lactobacillus* GG, *Bifidobacterium bifium* etc) early in the course of diarrhea is shown to reduce the duration of diarrhea and rotavirus shedding in affected patients (Saavedra and others 1994; Guandalini and others 2000; van Niel and others 2002).

In principle, the prevention of rotavirus infection can be achieved by avoiding exposures and fecal-oral spread (Anderson and Weber 2004). Contact with sick children and potentially contaminated food and water should be avoided, and contaminated objects and surfaces should be properly disinfected (Leung and others 2005). General measures such as personal hygiene and frequent hand washing may help control outbreaks in hospitals and child care center (Leung and others 2005). In addition, breast-feeding is encouraged, as it may be associated with milder disease in affected infants (Clemens and others 1993).

Fortunately, rotavirus infection is regarded as the single most frequent vaccine-preventable disease among children, and currently universal rotavirus vaccination is frequently used to control the disease (Widdowson and others 2005; Soriano-Gabarro and others 2006). Two effective rotavirus vaccines, RotaTeq (Merck) and Rotarix (GlaxoSmithKline) were

licensed for use in various countries of the world, beginning in 2004-2005 (Yen and others 2011). WHO recommends the routine immunization of all infants, and has introduced the rotavirus vaccine programs to countries with a high incidence of rotavirus mortality but lack of infrastructure or financial resources to develop such programs themselves (WHO 2009).

2.2.3 Epidemiological aspects of rotavirus disease

Human rotaviruses are mainly transmitted by faecal-oral route, person-to-person contact and the inhalation of airborne human rotaviruses or aerosols containing the viruses (WHO 2011b). In consequence, they can generate small epidemic outbreaks in all age groups, particularly within schools, hospitals, nursing homes, and care centers (Ruggeri and Fiore 2013). In addition, human rotaviruses are transmitted through faecally contaminated food and water, and thus results in occasional waterborne and foodborne outbreaks (WHO 2011b).

Earlier study on the seasonality of rotavirus disease found that rotavirus infection was certainly more common in the cooler months in temperate regions, whereas seasonal peaks of the infections can vary broadly and occur from autumn to spring in warmer tropical regions (Cook and others 1990). More recently, however, the seasonality of the rotavirus infection is shown to have stronger relation to the level of country development than latitude or geographic location (Pitzer and others 2011; Patel and others 2013). Patel and others (2013) found that poorer countries, particularly those in Africa, Asia, and South America have shown year-round circulation and had lesser seasonal variation in disease than more developed countries like Europe, North America and Oceania. In addition, one study by Pitzer and others (2011) suggested that the high birth rates and transmission rates typical of developing countries may be the reason for the relative lack of rotavirus seasonality observed in many tropical countries rather than being driven primarily by environmental conditions.

In accordance with increasing global burden of rotavirus disease and development of rotavirus vaccines, the introduction of rotaviruses surveillances program by WHO and others helps describing the diversity of rotavirus strains in different countries and their regions, and identifying the emerging strains (Chen and others 2012; Patel and others 2013). Currently, 27 G genotypes and 35 P genotypes have been described, and at least 73 G/P genotype combinations of group A rotaviruses have been detected (Matthijnssens and others 2012; Patton 2013). Globally, G1, G2, G3, G4 and G9 are the most prevalent VP7

serotypes; P[4], P[6] and P[8] are the most common prevalent VP4 genotypes (Chen and others 2012). Five rotavirus strains of G/P combination; G1P[8], G2P[4], G3P[8] , G4P[8] or G9P[8], are the major causes of children rotavirus diseases globally (Patel and others 2011). Among these strains, G1P[8] rotaviruses are consistently primary cause in North America, Austrailia and Europe (70% of infections), whereas less in South America, Asia and Africa (20-30%) (Soriano-Gabarro and others 2006; Iturriza-Gomara and others 2009). Recently, G12 have emerged and spread globally over the past decade (Matthijnssens and others 2009; Patton 2013).

In developing countries, uncommon G/P combinations may be also a frequent cause of disease in young children, and the types of uncommon strains varies from one region to another region (Armah and others 2010; Binka and others 2011). WHO surveillance program has revealed that predominant uncommon strains are G12P[8], G12P[6] in the Southeast Asia, G2P[6], G3P[6] and G1P[6] in the sub-Saharan Africa, G1P[4], G2P[8] in the Western Pacific, and G9P[4] in the Americas (WHO 2011a). The chance of rotavirus co-infection to generate reassortant viruses seems to be higher in developing countries than in developed countries, contributing the greater strain diversity in developing countries (Patton 2013).

2.3 Rotavirus in drinking water

2.3.1 Waterborne transmission

Human rotaviruses are excreted by patients in large quantities up to 10^{11} particles per gram of faeces for periods of about 8 days (WHO 2011b). It means that wastewater receiving faecal matter and any environments polluted with human faeces are likely to contain high numbers of human rotaviruses (Bosch 1998; WHO 2011b; Rosa and others 2012). A significant viral load can be released in effluent discharge and spread to the aquatic environment such as estuarine water, seawater and river (Rosa and others 2012). In consequence, all types of water including raw or treated sewage, river water, recreational water, and drinking water, are potential vehicles of virus transmission as described in Figure 4 (Bosch and others 2011). The high stability of rotaviruses in environmental water and their resistance to disinfection treatments may facilitate the waterborne transmission of rotaviruses (He and others 2009). In addition, rotaviruses are highly contagious and infectious dose is very low since as few as 10 particles can cause infection (Chen and others 2012). Ultimately, the consumption of contaminated drinking water or fruits and vegetables being in contacted with contaminated water poses public health risk, although

waterborne transmission of human rotaviruses is a not major route of exposure (Bosch and others 1998, 2008, 2011; WHO 2011b).

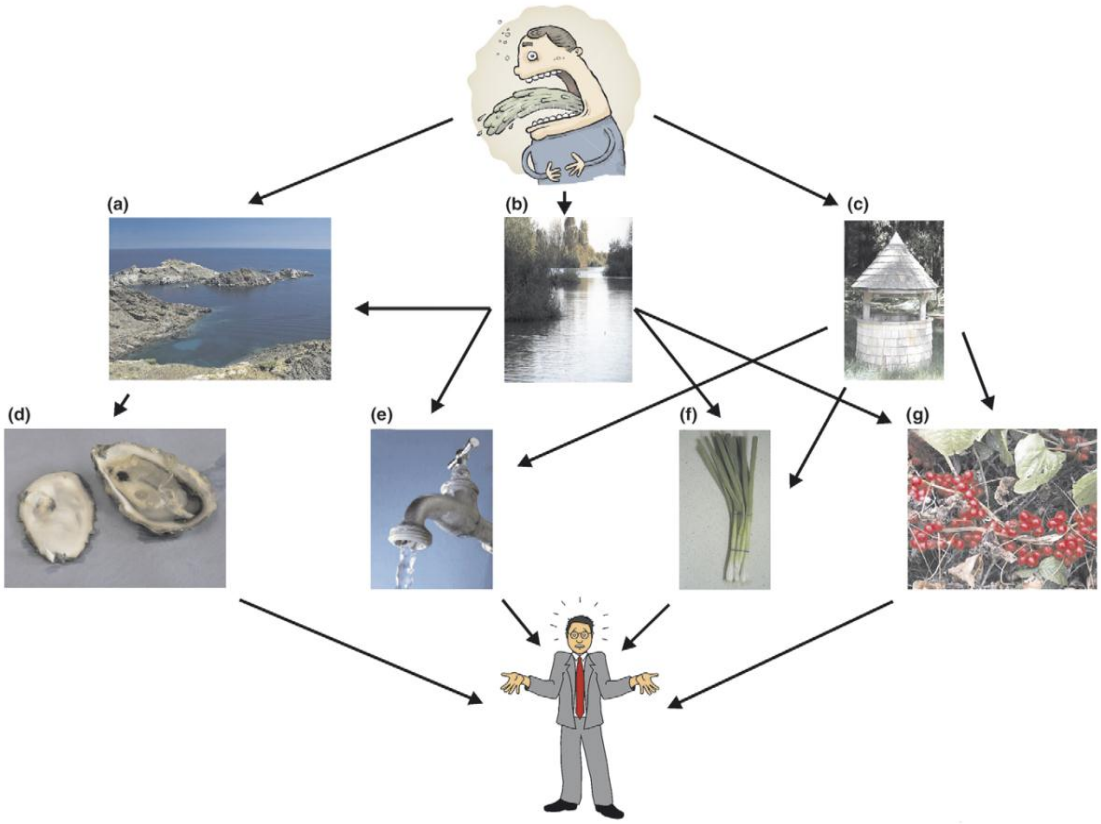

Figure 4. Possible routes of waterborne transmission of enteric viruses. Viruses are shed in extremely high numbers in the faeces and vomit of infected individuals. Pathogenic viruses are routinely introduced into the environment through the discharge of treated and untreated wastes. In consequence viral pathogens contaminate (a) marine environment, (b) fresh water and (c) ground water. Humans can be exposed to enteric viruses through various routes: (d) shellfish grown in polluted waters, (e) contaminated drinking water, (f) foods susceptible to be contaminated at the pre-harvest stage such as salad crops, lettuce, green onions and other greens; and (g) fruits such as raspberries and strawberries. Adapted from Bosch and others 2008.

2.3.2 Prevalence of rotavirus contamination in water

Numerous studies have reported the presence of rotaviruses in all types of water including wastewater, surface water, and in drinking water as well as in food. The high numbers of rotaviruses have been reported in wastewater. In one study in China, He and others (2011) screened a total 96 samples of influent and effluent wastewater taken from in three sewage treatment systems from November 2006 to October 2007 in order to know the presences of enteric viruses. The most frequently detected viruses were rotaviruses (32.3%), followed by astroviruses (6.3%) and noroviruses (3.1%). Specifically, rotaviruses were detected in 44.4% of influent samples, and in 25% of the effluent samples. Similarly, in another study in Norway, Myrmel and others (2006) examined the wastewater samples collected from the inlet and outlet of three sewage treatment plants, and found that rotaviruses were detected in 83%, 72%, and 38% of inlet samples and in 68%, 56%, and 36% of outlet

sample in three sewage treatment plants, respectively. In the study conducted in Germany, Pusch and others (2005) found that rotaviruses were detected in 3–24% of 123 treated wastewater samples collected from three sewage treatment plants. These studies demonstrated that untreated and treated wastewater may be a source of viral dissemination, and they are responsible for the environmental spread of rotaviruses.

The presence of rotaviruses has been also reported in surface water and drinking water. In one study in China, He and others (2012) collected a total 108 urban surface water samples from September 2007 to August 2007, and examined for the presence of enteric viruses. Among 63 virus strains identified, the most predominant viruses were rotaviruses (48%), followed by astroviruses (5.6%) and noroviruses (4.6%). In another study in Slovenia, Steyer and others (2011) screened surface water and drinking water for the presence of enteric viruses. Group A rotaviruses were detected in only 17.5% of the total 63 surface water samples, while noroviruses were more prevalent (41.3%) in surface water samples. On the other hand, among 72 drinking water samples, group A rotaviruses were the most prevalent (37.5%), followed by noroviruses (2.8%) and astroviruses (1.4%). These studies demonstrated the high prevalence of rotaviruses in surface water and drinking water, and highlighted the possibility to get rotavirus infection through the consumption of water.

Although rotaviruses were estimated to cause only 1% of all food-related illness and death (Mead and others 1999), the presence of rotaviruses has been occasionally reported in food. In the case of foodborne transmission, the main source of contamination is considered to be polluted water that has been in contact with food or inadequately treated or untreated sewage used for irrigation (Bosch and others 2008). In one study in Costa Rica, Hernandez and others (1997) investigated the presence of rotaviruses in lettuce bought in farm markets. Those samples collected during months (from December to January) of high incidence of rotavirus diarrhea were positive for rotavirus, and they suggested that the lettuce might have been contaminated with sewage. In addition, one study in Canada, Mattison and others (2010), they claimed the possible foodborne transmission of rotaviruses through the packaged leafy greens, although only 0.4% of samples were positive for rotaviruses. In another study in Canada, Brassard and others (2012) detected two positive samples of group A rotaviruses in 60 strawberries samples, and here irrigation water was suspected to be the source of contamination. These findings implied that the possible risk of rotavirus to contaminate food through waterborne transmission should be taken into account, even if the prevalence of rotavirus in food is expected to be relatively lower than that of other enteric viruses.

2.3.3 Waterborne outbreaks

Waterborne rotavirus infections are responsible for significant morbidity and mortality (Glass and others 2001; Villena and others 2003; Divizia and others 2004). Occasional waterborne outbreaks mainly due to the consumption of drinking water contaminated with rotaviruses have been reported (Table 2). Thus, the presence of human rotaviruses in drinking water is directly related to public health risk (WHO 2011b).

Table 2. Rotavirus related waterborne outbreaks due to the consumption of contaminated drinking water.

Month/Year	Place/Country	Etiological agents	Predominant symptoms	Reference
March 2012	Elassona, Greece	Rotavirus	*AGI; diarrhea, abdominal pain, vomiting and fever	Mellou and others 2014
November 2007	Nokia, Finland	Norovirus, Astrovirus Adenovirus, Rotavirus, Enterovirus	AGI; diarrhea and vomiting	Maunula and others 2009
November 2005	Malatya, Turkey	Rotavirus	AGI; diarrhea, abdominal pain, fever, and vomiting	Koroglu and others 2011
December 2000	Tirane, Albania	Rotavirus, Astrovirus, Adenovirus, Norovirus	AGI	Villena and others 2003
August 2000	Gourdon,France	Rotavirus, Norovirus Co-infection	AGI; diarrhea, abdominal pain and nausea	Gallay and others 2006
April 1994	Noormarkku, Finland	Norovirus, small round virus, Rotavirus	AGI; abdominal pain, severe vomiting, in some cases high fever, headache and diarrhea.	Kukkula and others 1997
March 1981	Eagle-Vail, Colorado, USA	Rotavirus	AGI; diarrhea and vomiting	Hopkins and others 1984

*AGI = acute gastrointestinal illness

2.4 Molecular detection of rotavirus in water

2.4.1 Current methods and challenges

Environmental virology research has been focused on aquatic environment including wastewater, surface water, and drinking water, mainly owing to public health concerns of viral related waterborne diseases and outbreaks (Bosch and others 2008; Gensberge and Kostic 2013). Historically, the virological analysis of environmental water samples has been challenging due to (i) the low concentration of target viruses; (ii) the presence of PCR inhibitors in water; (iii) diverse and evolving nature of viruses (Hamza and others 2011; Gensberge and Kostic 2013). Particularly, the environmental analysis of rotavirus has more difficulties and requires different methodological protocols than other enteric viruses (Ruggeri and Fiore 2013). Thus, there is a need for the reliable and reproducible analytical methods for the detection of waterborne viruses such as human rotavirus in environmental samples, in order to identify the infectious risk for public health and to reduce their impact (Rosa and Muscillo 2013).

The virological analysis of environmental water is a complex process that can be divided into two main steps: virus concentration and virus detection (Bosch and others 2008; Hamza and others 2011). As mentioned before, the concentration of viruses in water is usually very low, and they are distributed heterogeneously in environmental water samples (Rodriguez-Lazaro and others 2012). Thus, a desirable virus concentration method should be able to concentrate only viral particles while avoiding co-concentration of inhibitory compounds in water samples (Rosa and Muscillo 2013). Currently, three concentration techniques are commonly used: adsorption/elution, ultrafiltration, and ultracentrifugation (Rosa and Muscillo 2013). After the concentration of target virus from the samples, virus detection can be performed with either cell culture which is based on the observation of cytopathogenic effects (CPE) caused by viruses to cells, or molecular techniques such as PCR or qPCR assays which basically detect the target viral genomes by molecular amplification after viral nucleic acid extraction and purification (Bosch and others 2011; Rodriguez-Lazaro and others 2012).

Cell culture

Cell culture is the standard method to isolate human viruses in environmental samples based on the ability of viruses to produce visible cytopathogenic effects (CPE) (Rosa and Muscillo 2013). After infectious viruses are propagated in suitable cell culture, the

cytopathogenic effects (CPE) can be quantified with plaque assay (plaque forming unit PFU) or 50 % tissue culture infections dose ($TCID_{50}$) (Theron and Cloete 2002; Bosch and others 2011). However, the cell culture is basically difficult to perform and time-consuming, and more importantly, it is not universally applicable to all viruses since some viruses are non-cultivable or grown with difficulty (Yeh and others 2009; Rosa and Muscillo 2013). In addition, inoculated cell culture often deteriorates before the presence of CPE, making it difficult to obtain reliable and reproducible results (Ko and others 2003). Nevertheless, to date, it is considered to be the only reliable method to detect and quantify infectious viral particles (Yeh and others 2009; Bosch and others 2011).

Human rotaviruses are difficult to propagate as they are fastidious and may require more than 1 week to produce clear CPE (Li and others 2009). Fortunately, several cell lines have found to be efficient to some extent, for example, the cell-culture adapted rotavirus strains such as the human strain WA (Wyatt and others 1980) or the simian strain SA-11 (Estes and others 1979). However, cell culturing is not sufficiently sensitive for all human rotavirus strains, especially wild rotaviruses naturally contaminating water (Ruggeri and Fiore 2013).

Molecular techniques

With the development of molecular biology techniques, the application of PCR-based assays which detect the genome of target virus has considerably improved the ability to detect viruses in environmental samples (Mackay 2002). In brief, the PCR is a procedure by which specific sequence of DNA can be copied and amplified a billion-fold by exploiting DNA polymerase and using short sequence-specific primers (Valasek and others 2005). As PCR assay generally must use DNA as a template and some viral genomes such as rotaviruses are solely composed of RNA, reverse transcription-PCR (RT-PCR) assay is used (Valasek and others 2005). This assay utilizes reverse transcriptase which generates a complementary DNA (cDNA) from a RNA template and then the cDNA can be amplified by PCR (Bustin 2000; Valasek and others 2005). The relative amount of a given cDNA generated by reverse transcription is proportional to the relative amount of its RNA template (Valasek and others 2005). The RT-PCR assay has been applied for rotavirus detection in environmental samples in several studies (Gratacap-Cavallier and others 2000; He and others 2009; Yang and others 2011a). It has shown to have higher specificity and sensitivity for the detection of rotavirus compared to electron microscopy and immunoassays (Buesa and others 1996; Tang 2000). On the other hand, conventional PCR

assays are time-consuming, labor-intensive and non-quantitative, and they have substantial probability of cross-contamination due to post-PCR handling steps (Valasek and others 2005).

More recently, real-time quantitative PCR (qPCR) assay has been developed with the application of fluorescence techniques to the conventional PCR assay (Bustin 2000). The development of quantitative PCR (qPCR) assay has enabled rapid, sensitive and specific virus detection as well as quantitation of viral load (Bustin and others 2005; Valasek and others 2005). In principle, the qPCR assay integrates both amplification and detection by using fluorescent indicators such as double-stranded DNA dyes or fluorescently labelled probes, and instrumentations to detect emitted fluorescent signal (Wittwer and Farrar 2011). The amount of emitted fluorescence is proportional to the amount of PCR product (Klein 2002). Since the fluorescence level is detected after each cycle, it is possible to monitor the progress of PCR reaction in real-time and measure the quantity of PCR product during "exponential phase" in every cycle (Wittwer and Farrar 2011). Thus, the qPCR assay enables the accurate estimation of the quantity of initial template (Bustin 2000; Valasek and others 2005). Similarly in the RT-PCR assay, reliably generated cDNA from RNA is used as the template for qPCR (Valasek and others 2005).

In addition to the inherent quantitative potential of PCR, the qPCR or RT-qPCR assays represent technological advance over conventional PCR assays for several reasons: (i), high sensitivity as they have ability to detect less than 5 copies of target sequence and quantify the target sequence with a wide dynamic range (7–8 log units) (Klein 2002); (ii) minimized cross-contamination as they are performed in a close-tube reaction (Bustin and others 2005; Valasek and others 2005); and (iii) rapidness due to reduced cycle time and high-throughput automation system (Mackay and others 2002; Valasek and others 2005). The RT-qPCR assay has become the method of choice for the detection of RNA viruses, and currently, this approach is widely used in the field of food and environmental virology and continuously evolving (Yeh and others 2009; Bosch and others 2011; Rodriguez-Lazaro and others 2012). The RT-qPCR assay has been applied for rotavirus detection in environmental water samples in several studies (Verheyen and others 2009; Ganime and others 2012; Ye and others 2012).

However, qPCR or RT-qPCR assays have some limitations in routine virological analysis (Bosch and others 2008). The majority of these limitations are also present in conventional PCR or RT-PCR assays. First, they are susceptible to obtain either false-positive results

due to cross-contamination, or false-negative results due to inefficient nucleic acid extraction or due to the presence of inhibitory substance in RT or PCR reaction (Gassilloud and others 2003; Yeh and others 2009; Bosch and others 2011). It is generally known that RNA is extremely labile compared with DNA, and therefore isolation must be carefully performed to ensure both the integrity of the RNA itself and the removal of contaminating nucleases, genomic DNA, and RT or PCR inhibitors (Valasek and others 2005). In addition, quality control (QC) measures by using positive and negative controls are critical (Bosch and others 2011; Rodriguez-Lazaro and others 2012). Moreover, a careful selection for highly conserved sequences targeting primers and probes is required for effective detection and absolute quantification in spite of the genomic diversity of viruses and continuous emergence of new virus variants (Bosch and others 2008).

In addition, it is critical to know the information on the number of viral particles with infective capacity in the field of environmental virology (Rodriguez-Lazaro and others 2012). However, the detection of the viral genome by itself does not provide any information about the infectious nature of the viruses (Duizer and others 2004; Bosch and others 2008; Yeh and others 2009). In consequence, the PCR-based molecular methods often lead to false-positive results and overestimation of viral infectivity (Gassilloud and others 2003; Duizer and others 2004). Duizer and others (2004) found that for most disinfection methods applied at levels where viral infectivity could no longer detected, viral RNA remained detectable by PCR assay. They demonstrated that the detection of viral RNA using PCR-based assays underestimated the reduction in viral infectivity. Choi and Jian (2005) also observed the discrepancy between the high number of genome copies of viruses detected by RT-qPCR and absence of infectivity detected by cell culture. They suggested that PCR results significantly overestimated the occurrence of infectious viruses in environment. These studies demonstrated that positive PCR results do not allow a definitive evaluation of the infectious capability of the viral genomes detected, although negative PCR results obtained with well standardized quality controls and highly sensitive PCR assays can provide robust evidence for the absence of pathogens or indicators in the samples (Rodriguez-Lazaro and others 2012).

Overall, the detection of viral genomes, especially for non-cultivable viruses, may be necessary to identify infectious risk for the human population but it is not sufficient for assessment of infectious risk (Glassilloud and others 2003). Thus, utmost caution should be taken in directly extrapolating positive PCR results of human viruses to assess public health risks (Choi and Jiang 2005).

2.4.2 Promising methods to measure viral infectivity by PCR-based assays

Viral infectivity can be defined as the capacity of viruses to enter the host cell and use the cell resource to replicate and produce infectious viral particles, which may lead to infection and subsequent disease in the human host (Black 1996; Rodriguez and others 2009; Rodriguez-Lazaro and others 2012). For viral infectivity, the functional integrity of two components; viral capsid and viral genome, is required (Strauss and Strauss 2002; Rodriguez and others 2009). An undamaged viral capsid is critical for the initiation of a successful infection, while at the same time the replication and translation of the viral genome to viral proteins and enzymes are important for the successful production of new viral particles (Rodriguez and others 2009). PCR-based molecular assays have been used to determine the presence of amplifiable undamaged genome which may indicate the good condition of viral capsid protecting viral genome (Rodriguez and others 2009). However, current limitations of using PCR based assays to determine viral infectivity have led to the recent development of the PCR assays combined with other techniques such as (i) pre-sample treatments with dyes or enzymes, (ii) immunocapture of the virus from the sample, (iii) cell culture and (iv) oxidative stress marker.

Dye treatment prior to PCR-based assays (EMA- or PMA-PCR)

One of the promising approaches to measure viral infectivity is pre-treatment of viral sample with a viability dye, such as ethidium monoazide (EMA) or propidium monoazide (PMA), prior to PCR assay (Fittipaldi and others 2011). EMA is a photosensitive analog of ethidium bromide (EB) which has been used as a DNA intercalating agent (Yielding and others 1984). PMA is identical to propidium iodide (PI) except the presence of an additional azide group allowing cross-linkage to DNA upon light exposure (Nocker and others 2006). In theory, both dyes can ideally only penetrate membrane-compromised dead cells and suppress its amplification, but not intact cells (Fittipaldi and others 2012). The mechanism of amplification signal suppression is not fully understood (Fittipaldi and others 2012). One possible mechanism was suggested that the azide groups that both dyes have in common, may be converted into highly reactive nitrene radicals and they allow covalent cross-linkage to DNA upon light exposure (Fittipaldi and others 2012). Such a binding event is assumed to modify DNA and inhibit its amplification by PCR (Nocker and others 2006). On the other hand, the excess dyes may react with water molecules and may be converted into hydroxylamine, and in consequence they may be no longer reactive (Nocker and others 2008).

The concept of EMA-PCR assay was first introduced by Nogva and others (2003) to differentiate viable and dead cells in bacterial culture. The EMA-PCR assays were shown to selectively amplify and quantify target DNA of viable cells of bacteria in some studies (Rudi and others 2005; Wang and Levin 2006). However, EMA has shown to also penetrate to viable cells of some bacterial species in other studies (Nocker and Camper 2006; Kobayashi and others 2009). Few studies have applied the EMA-PCR assays on enteric viruses, and the effect of EMA treatment has shown to be different depending on the virus species: Graiver and others (2010) found the ineffective binding of EMA to avian influenza viral genome, while Kim and others (2011) claimed the potential of using EMA treatment for selective detection of polioviruses. The lack of specificity for intact cells or capsid has remained the greatest concern with the application of EMA treatment (Fittipaldi and others 2012).

As the alternative molecules of EMA, PMA was invented later by Nocker and others (2006), and the higher impermeability of PMA than EMA through intact cells of bacteria was shown in their study. Since their invention, the PMA-PCR assays have been successfully applied in a wide range of microorganisms including bacteria (Yang and others 2012; Kaushik and others 2013; Zhang and others 2013), bacterial spores (Mohapatra and others 2012), fungi (Vesper and others 2008), and yeast (Andorra and others 2010). More recently, few studies have employed the PMA-PCR assays to different types of viruses (Table 3).

PMA-PCR assay is considered to be a promising tool as it is easy and rapid to perform and it can provide viability information (Fittipaldi and others 2012). In addition, it can potentially applicable to non-cultivable viruses to examine viral infectivity (Hamza and others 2011). On the other hand, the generation of false-positive signals due to incomplete signal suppression remains to be the greatest concern with the application of PMA treatment (Fittipaldi and others 2012). For further evaluation of this assay, see the discussion section.

Table 3. Overview of the publications where PCR-based assays combined with PMA pre-treatment were employed to discriminate between infectious and inactivated viruses.

Detection method	Viruses	Inactivation method	Reference
PMA-RT-qPCR	Bacteriophage T4	Heating at 85 °C or 110 °C, Proteolysis	Fittipaldi and others 2010
PMA-RT-qPCR	Coxsackievirus, Poliovirus, Echovirus, Norwalk virus	Heating at 19 °C, 37 °C, or 72 °C, Hypochlorite.	Parshionikar and others 2010
PMA-RT-qPCR	Bacteriophage MS2, Murine norovirus	Heating at 72 °C or 80 °C	Kim and others 2012
PMA-RT-qPCR	Hepatitis A virus	Hypochlorite, High-pressure, Heating at 99 °C	Sanchez and others 2012
PMA&surfactants - RT-qPCR	Hepatitis A virus, Simian rotavirus, Human rotavirus	Heating 37 °C, 68°C, 72°C or 80 °C	Coudray-Meunier and others 2013

Enzymatic treatment prior to PCR-based assays

Another promising approach to assess viral infectivity is enzymatic pre-treatment of viral sample prior to PCR assays (Nuanualsuwan and Cliver 2002). This approach is based on the ability of viral capsid to protect the genomes from protease and nuclease. In principle, viral capsids of infectious viruses must be sufficiently intact to protect the viral genome from degradation. Nuanualsuwan and Cliver first proposed the pre-treatment of viral sample with both proteinase K and RNase prior to PCR to discriminate between infectious and inactivated viruses. They hypothesized that viral capsids of inactivated viruses can be more easily degraded by enzyme such as protease. Then the degraded capsids may allow unprotected nucleic acid to be exposed and degraded by nuclease, yielding a negative PCR result. On the other hand, intact capsids may protect nucleic acid from protease and nuclease, resulting in a positive PCR result. Later, this approach has been applied to different types of viruses (Table 4). Similarly as dye treatment, enzymatic treatment is easy and rapid to perform, and it can be potentially applicable to non-cultivable viruses to assess the viral infectivity (Hamza and others 2011). However, the capsid integrity alone as the criterion for viral infectivity may be limited (Pecson and others 2009; Hamza and others 2011). For further evaluation of this assay, see the discussion section.

Table 4. Overview of the publications where PCR-based assays combined with enzymatic pre-treatment were used to discriminate between infectious and inactivated viruses.

Detection method	Viruses	Inactivation method	Reference
Proteinase K and RNase-RT-PCR	Hepatitis A virus, Poliovirus, Feline Calicivirus	Ultraviolet light, Hypochlorite, Heating at 72 °C.	Nuanualsuwan and Cliver 2002
Proteinase K and RNase-RT-PCR	Human Picornavirus, Feline Calicivirus	Ultraviolet light, Hypochlorite, Heating at 37 °C, 72 °C	Nuanualsuwan and Cliver 2003
Proteinase K and RNase-RT-q PCR	Murine norovirus	Heating at 80 °C	Baert and others 2008
RNase-RT-q PCR	Feline Calicivirus, Human norovirus	Heating at 20 – 80 °C	Topping and others 2009
Proteinase K and RNase-RT-q PCR	Bacteriophage MS2	Ultraviolet light, Singlet oxygen, Heating at 72 °C	Pecson and others 2009
RNase-RT-q PCR	Hepatitis A virus	Hypochlorite, High-pressure, Heating at 99 °C	Sanchez and others 2012
Pronase and RNase-RT-q PCR	Human norovirus, Murine norovirus	Ultraviolet light	Rönnqvist and others 2014

Immunomagnetic separation prior to PCR-based assays (IMS-PCR)

Immunomagnetic separation technique utilizes paramagnetic beads coupled to a virus-specific antibody targeting viral antigen, allowing the separation of virus from contaminating materials and virus concentration in a single step (Gilpatrick and others 2000). The combination of immunomagnetic separation method and PCR assays (IMS-PCR) was first developed by Grinde and others (1995) for rotavirus detection. They found that this assay provided a better correlation with viral infectivity than either method alone. This assay enabled the detection of target viral genome packed in capsid proteins, not just the presence of proteins or of naked viral genome (Grinde and others 1995). Later, this method has been applied for the detection of enteric viruses such as enterovirus (Casas and Sunen 2002), hepatitis A virus (Casas and Sunen 2002), human norovirus (Gilpatrick and others 2000; Myrmel and others 2000), human rotavirus (Grinde and others 1995), and simian rotavirus (Casas and Sunen 2002; Yang and others 2011b).

Casas and Sunen (2002) suggested that this method was relatively rapid and easy to perform and it enabled efficient, sensitive and specific detections of enteric viruses in environmental samples despite the presence of complex inhibitory substances. In addition, Yang (2011b) found that this assay had higher virus recovery efficiency by removing the PCR inhibitors in complex sewage concentrates, and the results showed a good correlation with cell culture. On the other hand, this method seems to be highly dependent on antigenic properties of the viral capsids, so that the conformational changes in the viral proteins could inhibit the interaction with antibodies (Rodriguez and others 2009). In addition, it may require a specific assay for each virus strain since certain antibody may not able to target all possible strains of the viruses (Hamza and others 2011).

Integrated cell culture-PCR-based assays (ICC-PCR)

Integrated cell culture with PCR (ICC-PCR) is an approach to overcome most of the disadvantages of both cell culture assay and PCR assay (Rodriguez and others 2009). Detection is based on an initial biological amplification of viral nucleic acid using cell culture and followed by PCR amplification (Hamza and others 2011). In consequence, it enables the selective enumeration of infectious virus with rapid detection (Rigotto and others 2010). Reynolds and others (1996) first introduced the ICC-PCR assay for detection of human enteric viruses in environmental samples. Since then, the ICC-PCR assays have been applied for the detection of human enteric viruses including adenovirus (Greening and others 2002; Cheong and others 2009; Amdiouni and others 2011), enterovirus (Reynolds and others 2001; Greening and others 2002; Shieh and others 2008), hepatitis A virus (Reynolds and others 2001), poliovirus (Blackmer and others 2000), human rotavirus (Rutjes and others 2009; Li and others 2009, 2010, 2011), and simian rotavirus (Li and others 2009, 2010, 2011) in environmental water samples.

Reynolds and others (2001) found that ICC-PCR was useful for the evaluation of viral infectivity in accordance with cell culture assay with shorter incubation time. In addition, Li and others (2009) showed that ICC-RT-qPCR was more effective, sensitive and faster than direct RT-PCR for rotavirus detection with the information of infectivity. Moreover, a cell culture step may eliminate or reduce inhibitory compounds in environmental samples (Rodriguez and others 2009; Gensberger and Kostic 2013). However, it may require multiple cell lines and it is not applicable for non-cultivable viruses, such as human norovirus (Hamza and others 2011).

Oxidative stress marker

A novel approach to assess the infectivity of non-cultivable viruses was recently proposed by Sano and others (2010). In theory, some amino acids such as lysine, arginine, proline, and threonine can form carbonyl groups by the oxidative reaction with different chemicals, and then this carbonylation on protein molecules could result in the loss of protein functions (Levine 2002; Temple and others 2006). Non-enveloped enteric viruses could be injured by exogenous stress in natural environment, and damages on viral capsid would lead to loss of infectivity (Sano and others 2010). Accordingly, cumulative carbonyl groups on viral particles created by oxidative stress may be detected by labeling with a biotin that can bind to the carbonyl group, and then damaged virus particles (biotinylated) could be separated from intact virions (non-biotinylated) using avidin-immobilized affinity chromatography (Mirzaei and Regneler 2005; Tojo and others 2013). Thus intact and damaged virions can be separately quantified by PCR assays (Tojo and others 2013). This approach has been only applied to the detection of human astrovirus and norovirus (Sano and others 2010) and rhesus rotavirus (Tojo and others 2013).

Sano (2010) claimed that the oxidative products on viral capsid proteins might be quantitatively detected as an indication of viral particle integrity, which has a significant correlation with viral infectivity, and thus the direct detection of oxidative damage by this approach seemed to be a powerful tool for the evaluation of viral infectivity of non-cultivable viruses. Later study by Tojo (2013) confirmed the ability to determine the reduction level of viral infectivity using this method, and the infectivity reduction level was equivalent to that achieved using the plaque assay. However, the absence of oxidative damage may not ensure that viruses are still infectious, in case other mechanisms have led to virus inactivation in the environment, and furthermore it may not be applicable in routine basis due to the high cost (Hamza and others 2011).

3. EXPERIMENTAL RESEARCH

3.1 Aims of the present study

The aim of the study was to develop a reliable molecular method for rotavirus detection with information on viral infectivity, and which may also contribute to the development of molecular detection methods for non-cultivable health-significant viruses such as human norovirus. The specific aim was to evaluate the potential of using RT-qPCR assay combined with PMA or RNase treatment to assess the infectivity of human rotavirus (HRV), in comparison with using RT-qPCR assay without any pre-treatment. The cell culture-based infectivity assay was used as a reference method to measure the infectivity of HRV. It was hypothesized that using RT-qPCR assay with pre-treatments would be able to selectively distinguish between infectious and thermally-treated HRV.

3.2 Materials and methods

3.2.1 Virus and host cell

MA-104 African green monkey epithelial cell line (ATCC® CRL-2378.1) was obtained from Professor Lennart Svensson at the Linköping University (Linköping, Sweden). MA-104 cells were grown in Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich Co. Saint Louis, MO, USA), containing 10 % heat-inactivated foetal bovine serums (FBS) (Thermo Fisher Scientific Inc. Waltham, MA, USA), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) solution (Sigma-Aldrich Co.) and 1 % glutamine-penicillin streptomycin (Sigma-Aldrich Co.). The cells were grown at 37 °C in an atmosphere containing 5 % CO_2. Cells from passage 5 to passage 35 were used for the experiments.

Human Rotavirus (HRV) WA strain G1P[8] was obtained from Professor Lennart Svensson at the Linköping University (Linköping, Sweden), and was propagated in MA-104 cells. To produce virus stock, HRV WA strain was cultivated on confluent (80 %) cell monolayer for 2 – 3 days, and after appearance of the cytopathogenic effect (CPE) using a light microscope, the infected cells were frozen and thawed three times and then centrifuged at 4.5 x 10^3 g for 10 minutes at 4 °C to remove residual debris. The supernatant was subjected to ultrafiltration (Amicon Ultra-15; Millipore, Billerica, MA, USA) at 4.5 x 10^3 g for 10 minutes at 4 °C and the supernatant from the ultrafiltration unit was recovered, and adjusted to 2 ml with 1 x PBS, and stored in aliquots at – 70 °C.

The original HRV stock (containing 3.29 x 10^5 $TCID_{50}$/ml) produced as above was used in cell culture-based infectivity assay. Two different conditions of the HRV stocks were prepared for RT-qPCR assay as following; HRV stock A (concentration of 3.29 x 10^3 $TCID_{50}$/ml) which was diluted to 10^{-2} of the original virus stock due to the much higher sensitivity of RT-qPCR assay than cell culture-based infectivity assay; and HRV stock B (concentration not measured) which was diluted 10^{-2} of the original virus stock and subjected to RNase treatment (described in detail in section 3.2.6) in order to remove the possible presence of external RNA. Figure 5 describes the flow chart of experiments.

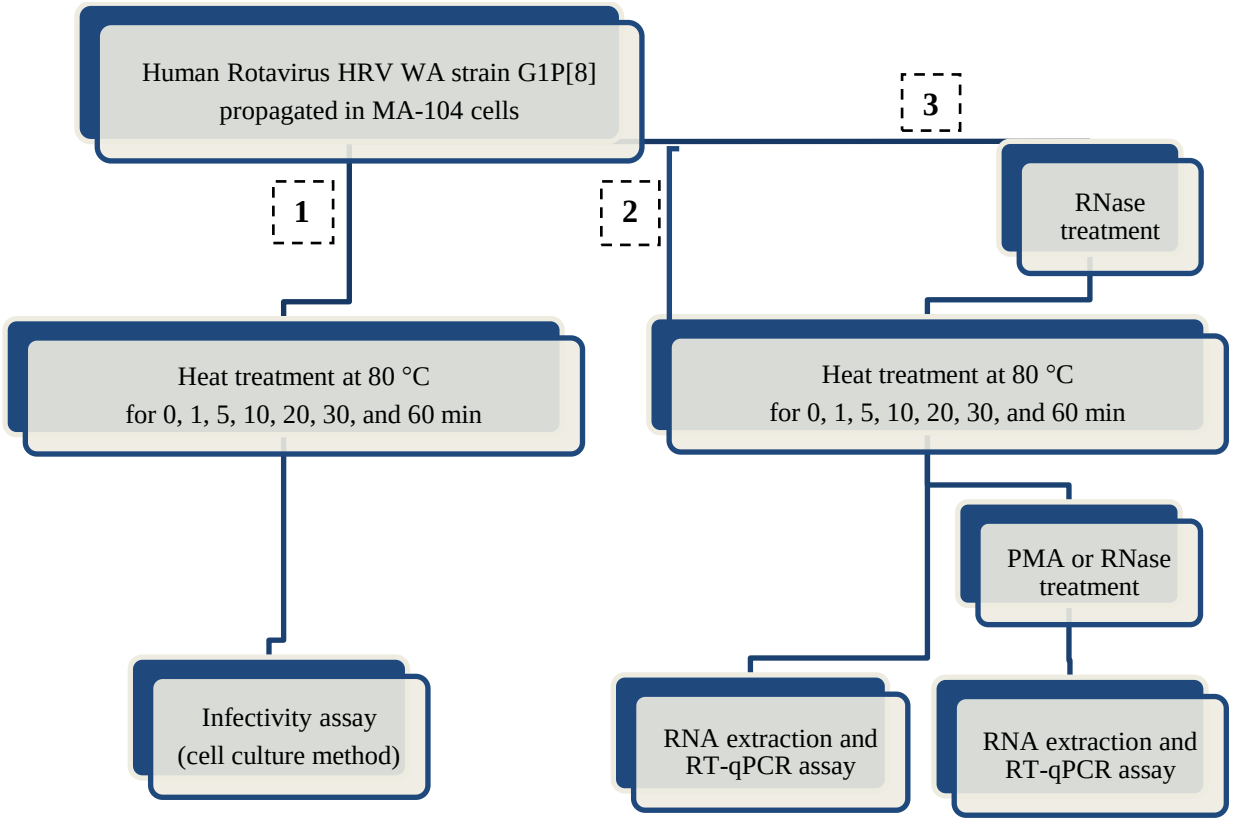

Figure 5. Flow chart of the experimental protocols; (1) original HRV stock (containing 3.29 x 10^5 $TCID_{50}$/ml) was thermally treated at 80 °C for different time points, and the infectivity was measured by cell culture-based infectivity assay, (2) HRV stock A (containing 3.29 x 10^3 $TCID_{50}$/ml) was thermally treated at 80 °C for different time points, and the virus titer was measured by RT-qPCR assay, RT-qPCR assay with PMA or RNase treatment, and (3) HRV stock B was thermally treated at 80 °C for different time points, and the virus titer was measured by RT-qPCR assay, RT-qPCR assay with PMA or RNase treatment.

3.2.2 Infectivity assay

The infectivity of original HRV stock was measured by determining the 50 % tissue culture infectious dose ($TCID_{50}$). In brief, MA 104 cells were diluted in EMEM containing 10 % FBS to a concentration of 1.0 x 10^6 cells/ml, and 500 µl of cell suspension was seeded into each well in a 24-well plate. After 24 h of incubation at 37 °C under 5 % CO_2, each well was washed with PBS once, and EMEM containing 0 % FBS was added. After 24 h of incubation, 200 µl of each 10-fold serial dilution of trypsin-activated HRV were added to four parallel wells on the plate per dilution. The plates were incubated at 37 °C under 5 % CO_2 and checked daily for the presence of CPE using a light microscope. The wells with CPE were recorded as positive. The final recording was performed after 7 days and $TCID_{50}$/ml was calculated using by Spearman and Kärber formula as described in Hierholzer and Killington (1996).

3.2.3 PMA treatment

PMA (propidium monoazide) was purchased from Biotium, Hayward, CA, USA at 20 mM in H_2O and diluted in sterile H_2O to obtain the solutions used in this study. The PMA solution was stored at – 20 °C in the dark. PMA was added to aliquots of 140 µl of HRV stock A or B to chosen concentrations, and incubated for 5 min in the dark at room temperature prior to light exposure for 15 min using the PMA-Lite LED (Light-emitting diode) Photolysis Device (Biotium, Hayward, CA, USA).

Prior to the optimal concentration (100 µM) of PMA being chosen for further studies, the effects of light exposure and different concentrations (50 and 100 µM) of PMA on infectious HRV stock A (3.29 x 10^3 $TCID_{50}$/ml) were preliminarily tested. Two different final concentrations of PMA were added to infectious HRV stock A. HRV stock A without PMA treatment was used as a control. Two identical sets of samples were prepared: one set of samples was exposed to light and the other set was not.

3.2.4 PMA treatment of viral RNA

The binding of PMA to viral RNA was tested to check whether the final concentration of 100 µM was sufficient enough to bind viral RNA and inhibit its amplification by RT-q assay. In detail, viral RNA was extracted from six aliquots of 140 µl of HRV stock A and eluted in 60 µl of elution buffer, according to the manufacturer's instructions with QIAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). The eluted viral RNAs were pooled to obtain homogeneous RNA stock. First, three different viral RNA samples were prepared as

following: one sample without any treatment, another one treated with PMA at a final concentration of 100 µM followed by light exposure, and the other treated with PMA at a final concentration of 100 µM followed by no light exposure. In addition, in order to remove the potential inhibitory effect of unbound PMA to RT-qPCR assay, identical samples were prepared as above, but then the samples were purified by using QIAquick PCR purification kit according to manufacturer's instructions. Finally, all samples were analyzed with RT-qPCR assay.

3.2.5 RNase treatment

RNase was purchased from Roche Diagnostics, Indianapolis, IN, USA and diluted in sterile Tris-HCI, H_2O and glyserol to obtain the solutions used in this study. The RNase solution was stored at – 20 °C. The chosen doses of RNase were added to aliquots of 140 µl of HRV stock A and B, and incubated at 37 °C for 30 min, after which 80 U of RNase inhibitor (RiboLock, Thermo Fisher Scientific Inc, Waltham, MA, USA) was added.

Prior to the optimal dose (0.08 mg) of RNase being chosen for further studies, the effects of different doses (0.04 and 0.08 mg) of RNase on infectious HRV stock A (3.29×10^3 $TCID_{50}$/ml) was preliminarily tested. Two different doses of RNase were added to the HRV stock A. HRV stock A without RNase treatment was used as a control.

3.2.6 Heat treatment

Heat treatment was conducted to study the potential of using PMA or RNase treatment to discriminate between infectious and thermally-treated HRV. In the preliminary tests, heat treatment at 37, 80, and 97 °C was selected to monitor the potential of both pre-treatments. HRV stock A (3.29×10^3 $TCID_{50}$/ml) was incubated for 30 min at each temperature using a dry bath set. For PMA experiment, the effects of different concentrations (50 and 100 µM) of PMA on thermally-treated HRV stock A were tested. Two different final concentrations of PMA were added to thermally-treated HRV stock A. The HRV stock A without PMA treatment was used as a control. For RNase experiment, the effects of different doses (0.04 and 0.08 mg) of RNase on thermally-treated HRV stock A were tested. Two different doses of RNase were added to thermally-treated HRV stock A. The HRV stock A without RNase treatment was used as a control.

Heat treatment at 80 °C was chosen for further studies. First, the original HRV stock (3.29×10^5 $TCID_{50}$/ml) was thermally treated by using a dry bath set at 80 °C for 0, 1, 5, 10, 20, 30, and 60 min, after which the samples were immediately placed on ice. The infectivity of

the thermally-treated HRV stocks at different time points was measured using infectivity assay as already described in section 3.2.2 in order to obtain thermal inactivation curve at 80 °C.

Second, HRV stock A (3.29 x 10^3 $TCID_{50}$/ml) was thermally treated as above. For PMA experiment, two identical HRV stock A samples were prepared at each time point; one sample was treated with 100 µM of PMA while the other was not. All samples were incubated for 5 min in the dark at room temperature and then exposed to light for 15 min. For RNase experiment, two HRV stock A samples were prepared at each time point; one sample was treated with 0.08 mg of RNase while the other was not. All samples were incubated for 30 min at 37 °C, and after which 80 U of RNase inhibitor was added only to the samples with RNase treatment. In each experiment, the non-pre-treated HRV stock A stored at 4 °C was used as a control. Finally, all samples were subjected to RNA extraction and analyzed with RT-qPCR assay. Each experiment was performed twice.

Lastly, HRV stock B was prepared after the addition of RNase (0.08 mg) to aliquots of 140 µl of HRV stock A and incubated at 37 °C for 30 min, after which 80 U of RNase inhibitor was added. A control was prepared by adding sterile H_2O instead of RNase and RNase inhibitor. HRV stock B was also thermally treated as above. PMA and RNase experiments were performed as described above. In each experiment, the non-pre-treated HRV stock B stored at 4 °C was used as a control. Finally, all samples were subjected to RNA extraction and analyzed with RT-qPCR assay. Each experiment was performed twice.

3.2.7 Viral RNA extraction

RNA extraction was performed according to the manufactures' instructions with QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Aliquots of 140 µl of each sample were applied for nucleic acid extraction, and nucleic acids were eluted in 60 µl of elution buffer and stored at – 20 °C.

3.2.8 VP2 gene specific primers and probes

The specific primers and probes targeting VP2 gene of HRV WA strain G1P[8] was used (Gutierrez-Aguirre and others 2008). The sequence of the primer pairs and probe was as follows: the forward primer (VP2-F2): 5'-CAGACACGGTTGAACCCATTAA-3, the reverse primer (VP2-R1): 5'-GTTGGCGTTTACAGTTCGTTCAT-3' and the VP2 Taqman probe: 5'-FAM-ATGCGCATRTTRTCAAAHGCAA-MGB-3'. The target gene sequence can be found with accession numbers X14942.1 in Nucleotide Database, in

National Center for Biotechnology Information (NCBI, Bethesda MD, USA). It generated amplification products of 84 bp.

3.2.9 RT-qPCR assay

One step RT-qPCR amplifications were performed in duplicate using Rotor-gene 3000 thermal cycler (Corbett Life science, Sydney, Australia). Each reaction was performed in a 20 µl reaction mixture containing 5 µl of template, 0.9 µM each forward and reserve primers (VP2-F2 and VP2-R1), 0.26 µM of VP2 Taqman probe, 10 µl of 1x QuantiTect Probe RT-PCR Master Mix (QIAGEN, Hilden, Germany), 0.2 µl of QuantiTect RT Mix (QIAGEN, Hilden, Germany), and 2.4 µl of RNase-Free water. The reaction conditions were as follows: reverse transcription of RNA for 60 min at 50 °C followed by PCR initial activation step for 10 min at 95 °C, and finally the 45 cycles of 2-step cycling step including; denaturation for 15 sec at 95 °C and combined annealing and extension for 60 sec at 60 °C.

The PCR titer of HRV stock A was measured using a standard curve. The standard curve was generated by amplifying 10-fold serial dilutions of original HRV stock from 10^{-2} to 10^{-8} by RT-qPCR assay in duplicate. The quantification cycle (Cq) at which target gene was amplified at exponential phase was determined by fluorescence threshold of 0.03. The Cq obtained from each dilution was used to plot the standard curve in the way that the highest dilution (10^{-7}) showing a positive results with Cq (< 40) was assigned a concentration of 10 RT-qPCR detectable virus units (PCR-units) and progressively 10-fold higher values to the lower dilutions, respectively. The equation of linear regression and the correlation coefficient (r^2) values were then obtained for the standard curve, and from the slope of the regression curve, PCR amplification efficiency was calculated according to following equation: $E = 10^{(-1/slope)} - 1$, where a value of 1 corresponds to 100 % efficiency. The PCR titer of unknown samples was determined by plotting the corresponding Cq value against the standard curve obtained as above. All unknown samples were analyzed with RT-qPCR assay in duplicates, and in every set of samples, two positive controls, one negative control for RNA extraction, one negative PCR control and one non-template control (NTC) were included.

3.2.10 Statistical analysis

Student's *t* test was used to evaluate the significance of difference among the mean numbers of virus titers between treated and non-treated samples, with a significance level of $P < 0.05$ (Microsoft Office Excel, Microsoft, Redmond, USA).

3.3 Results

3.3.1 Standard curve

The standard curve of HRV RT-qPCR assay was generated (Figure 6). The last dilution 10^{-7} showing positive Cq value (< 40) was assigned to the concentration of 10 RT-qPCR units which was the lowest level of detection per each reaction. As each reaction contained 5 µl of template, the detection limit of RT-qPCR assay was estimated to be 2 PCR-units per 1 µl of template. The PCR titer of HRV stock A was determined as 1.0 x 10^6 PCR-units per 5 µl of template. The linear dynamic ranges of detection were from 10^1 to 10^6 PCR-units. The linear regression curve was obtained using following equation: $y = -4.373\,x + 43.742$. The correlation coefficient (r^2) was 0.983 and the amplification efficiency was 0.69.

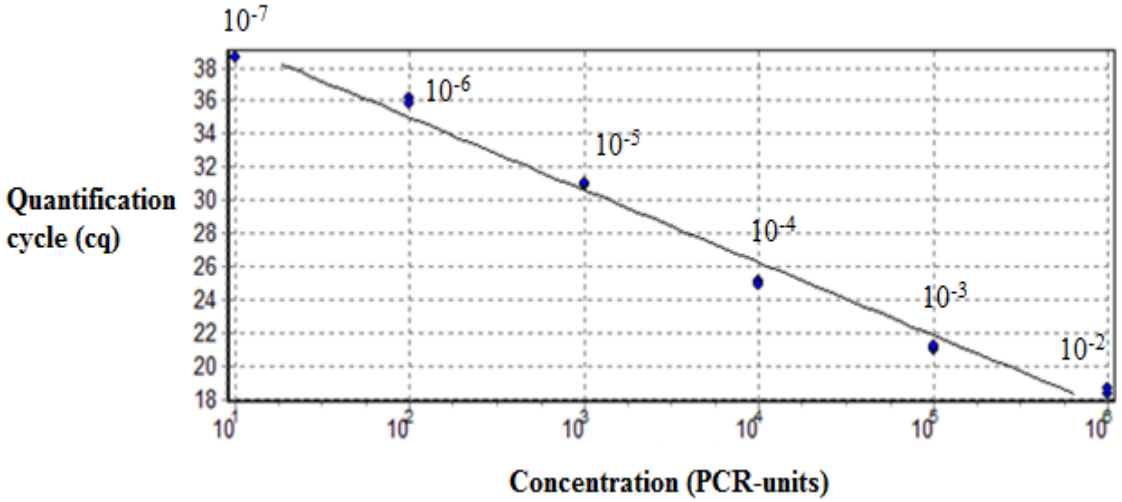

Figure 6. Standard curve of HRV RT-qPCR assay. The Cq obtained from each dilution was used to plot a standard curve in the way that the highest diluton (10^{-7}) with a Cq (< 40) was assigned a concentration of 10 PCR-units per 5 µl, and progressively, 10-fold higher values to the lower dilutions 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} respectively.

3.3.2 Validation of PMA treatment

The effects of light exposure and different PMA concentrations (50 and 100 µM) on infectious HRV stock A were investigated (Table 5). The effect of light exposure alone on infectious HRV stock A was negligible. PMA treatment without light exposure to infectious HRV stock A had no effect on virus titer detected by RT-qPCR assay regardless

of the PMA concentrations. On the other hand, after PMA treatment upon light exposure, the virus titer of infectious HRV stock A was decreased by slightly more than 1 log_{10} PCR-unit. The reduction level was slightly higher with PMA at a final concentration of 100 µM than at 50 µM.

Table 5. The effects of light exposure and different PMA concentrations (50 and 100 µM) on infectious HRV stock A.

	Level of HRV stock A (3.29×10^3 $TCID_{50}$/ml)			
	without light exposure		with light exposure	
	Quantification[1] Log_{10} (PCR-units)	Reduction[2]	Quantification[1] Log_{10} (PCR-units)	Reduction[2]
Infectious control	5,70 ± 0,05		5,66 ± 0,09	
Infectious + 50 µM PMA	5,72 ± 0,01	0,01	4,50 ± 0,33	1,16
Infectious + 100 µM PMA	5,71 ± 0,08	0,00	4,37 ± 0,34	1,29

1. HRV titers (PCR-units) were calculated by plotting the samples against the standard curve, and standard deviations were calculated among the biological replicates.
2. Reduction in quantification between infectious viruses before and after PMA treatment.

The effects of different PMA concentrations (50 and 100 µM) were preliminarily tested on thermally-treated HRV stock A at three different temperatures (37, 80, and 97 °C) for 30 min (Table 6). PMA treatment to thermally-treated HRV stock A decreased the virus titers about 1 log_{10} PCR-unit regardless of temperature (data at 97 °C not shown). The reduction level was slightly higher with PMA at a final concentration of 100 µM than 50 µM. Even after PMA treatment (100 µM), the titer of thermally-treated HRV stock A at 80 °C for 30 min (4.49 log_{10} PCR-units) was still as high as the titer of infectious HRV stock A (4.37 log_{10} PCR-units) (Table 5).

Table 6. The effects of PMA concentrations (50 and 100 µM) on thermally-treated HRV stock A.

	Level of HRV stock A (3.29×10^3 $TCID_{50}$/ml)			
	37 °C for 30 min		80 °C for 30 min	
	Quantification[1] Log_{10} (PCR-units)	Reduction[2]	Quantification[1] Log_{10} (PCR-units)	Reduction[2]
Thermally-treated control	5,61		5,70	
Thermally-treated + 50 µM PMA	4,67	0,94	4,80	0,90
Thermally-treated + 100 µM PMA	4,44	1,13	4,49	1,21

1. HRV titers (PCR-units) were calculated by plotting the samples against the standard curve, and standard deviations were calculated among the biological replicates.
2. Reduction in quantification between inactivated viruses before and after PMA treatment.

The binding of PMA (100 µM) to extracted viral RNA was investigated (Table 7). PMA treatment to non-purified viral RNA upon light exposure decreased virus titer by 4 log_{10} PCR-units. After RNA purification with QiaQuick PCR purification kit, the reduction level was decreased by 3 log_{10} PCR-units. In addition, PMA treatment to non-purified viral RNA without light exposure decreased virus titer by 5.12 log_{10} PCR-units, while the virus titer rendered similar as the titer of control viral sample after the purification.

Table 7. The binding of PMA on viral RNA with and without purification.

	Level of HRV stock A (3.29 x 10^3 $TCID_{50}$/ml)			
	Without purification		With purification	
	Quantification[1] Log_{10} (PCR-units)	Reduction[2]	Quantification[1] Log_{10} (PCR-units)	Reduction[2]
Viral RNA control	5,50 ± 0,04		5,47 ± 0,00	
Viral RNA + 100 µM PMA[3]	1,42 ± 0,29	4,08	2,45 ±0,22	3,02
Viral RNA + 100 µM PMA[4]	0,38 ± 0,54	5,12	5,36 ±0,05	0,11

1. HRV titers (PCR-units) were calculated by plotting the samples against the standard curve, and standard deviations were calculated among the biological replicates.
2. Reduction in quantification between viral RNA before and after PMA treatment.
3. With light exposure and analyzed with RT-qPCR assay.
4. Without light exposure and analyzed with RT-qPCR assay.

3.3.3 Validation of RNase treatment

The effects of different RNase doses (0.04 and 0.08 mg) on infectious HRV stock A were evaluated (Table 8). RNase treatment decreased virus titers slightly more than 1 log_{10} PCR-unit. The reduction level was slightly higher with 0.08 mg of RNase than 0.04 mg.

Table 8. The effects of two different doses (0.04 and 0.08 mg) of RNase on infectious HRV stock A.

	Level of HRV stock A (3.29 x 10^3 $TCID_{50}$/ml)	
	Quantification[1] Log_{10} (PCR-units)	Reduction[2]
Infectious control	5,65 ± 0,14	
Infectious + 0,04 mg RNase	4,61 ± 0,37	1,04
Infectious + 0,08 mg RNase	4,39 ± 0,62	1,26

1. HRV titers (PCR-units) were calculated by plotting the samples against the standard curve, and standard deviations were calculated among the biological replicates.
2. Reduction in quantification between infectious virus before and after RNase treatment.

The effects of different RNase doses (0.04 and 0.08 mg) were preliminarily tested on thermally-treated HRV stock A at different temperature (37, 80, and 97 °C) for 30 min (Table 9). RNase treatment decreased the titer of virus incubated at 37 °C for 30 min about 1 log_{10} PCR-unit. On the other hand, the titer of thermally-treated virus at 80 °C for 30 min

was decreased by 3 log_{10} PCR-units, and the similar reduction level was also observed for thermally-treated virus at 97 °C for 30 min (data not shown). The reduction levels of virus titer were similar for both RNase doses (0.04 and 0.08 mg)

Table 9. The effects of two different doses (0.04 and 0.08 mg) of RNase on thermally-treated HRV stock A.

	Level of HRV (3.29 x 10^3 $TCID_{50}$/ml)			
	37 °C for 30 min		80 °C for 30 min	
	Quantification[1] Log_{10} (PCR-units)	Reduction[2]	Quantification[1] Log_{10} (PCR-units)	Reduction[2]
Thermally-treated control	5,64		5,56	
Thermally-treated + 0,04 mg RNase	4,59	1,05	2,60	2,95
Thermally-treated + 0,08 mg RNase	4,51	1,13	2,62	2,94

1. HRV titers (PCR-units) were calculated by plotting the samples against the standard curve, and standard deviations were calculated among the biological replicates.
2. Reduction in quantification between inactivated viruses before and after PMA treatment.

3.3.4 Thermal inactivation curve

The infectivity of original HRV stock was measured by cell culture-based infectivity assay after heat treatment at 80 °C at different time points, and thermal inactivation curve was generated (Figure 7). The initial infectivity of HRV without any heat treatment was 3.29 x 10^5 $TCID_{50}$/ml. After 1 min, the infectivity level of HRV was dramatically decreased to 1.04 x 10 $TCID_{50}$/ml. After 5 min, HRV was completely inactivated. The detection limit of infectivity assay was estimated to be 2.18 $TCID_{50}$/ml.

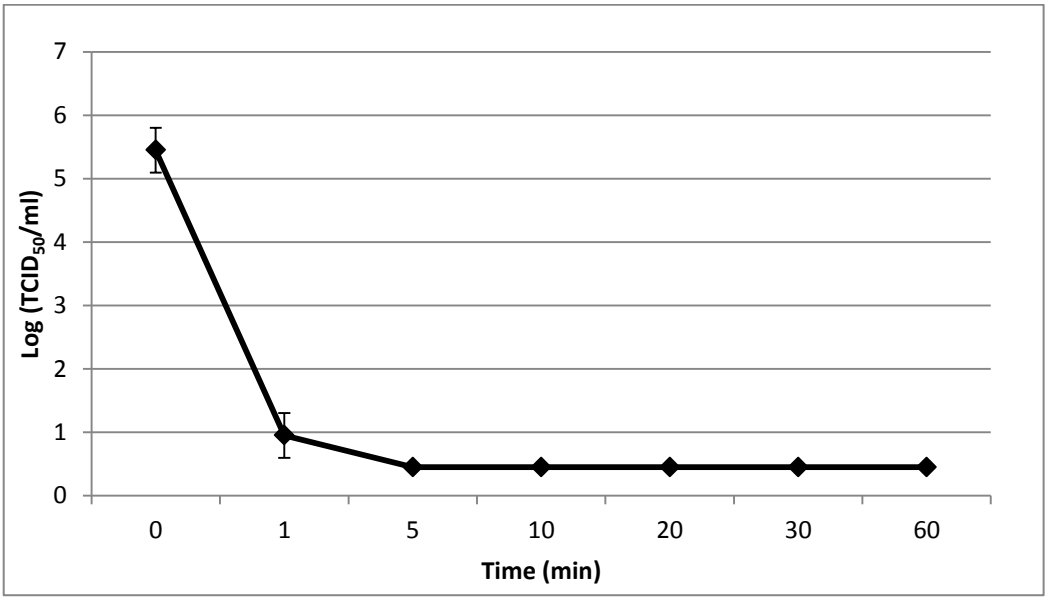

Figure 7. Thermal inactivation curve of original HRV stock at 80°C. x (horizontal) axis stands for different time points applied at 80 °C, and y (vertical) axis stands for the infectivity level of virus by log ($TCID_{50}$/ml).

3.3.5 PMA treatment to monitor thermal inactivation of HRV stock A

The potential of PMA treatment to distinguish infectious and thermally-treated HRV stock A was investigated (Figure 8). Using RT-qPCR assay, similar virus titers (black column in Figure 8) were observed for both infectious and thermally-treated HRV regardless of inactivation time. On the other hand, virus titers measured by RT-qPCR assay with PMA treatment (gray column in Figure 8) were significantly ($P < 0.05$) different from the titers measured by RT-qPCR assay alone. After PMA treatment, the titer of infectious HRV was decreased by 1.29 $\log_{10}$ PCR-units. The reduction levels were similar for thermally-treated HRV regardless of inactivation time (ranges from 1.04 to 1.24 $\log_{10}$ PCR-units).

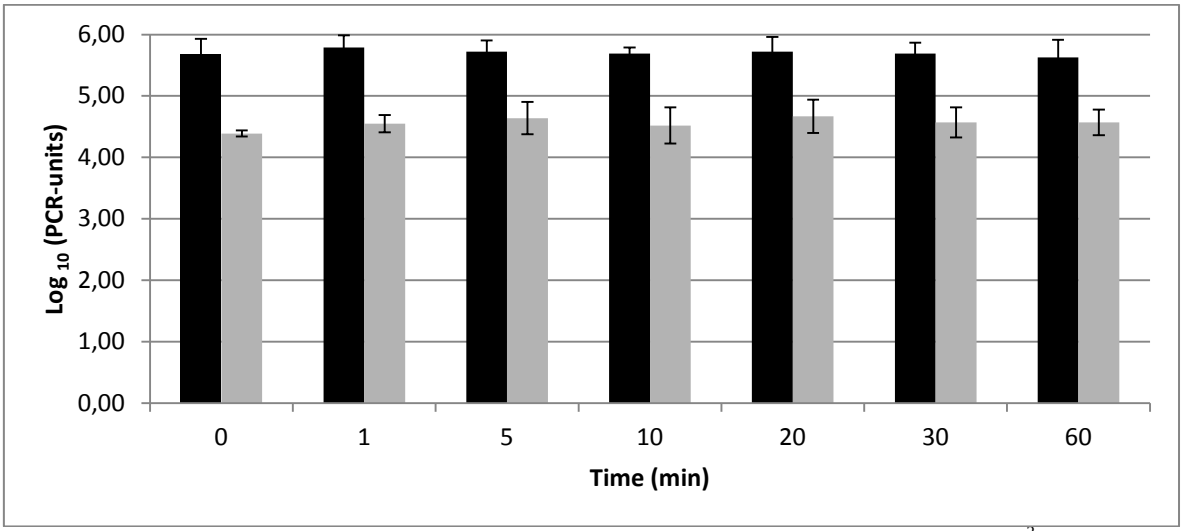

Figure 8. Quantification of infectious and thermally-treated HRV stock A (containing 3.29 x 10^3 $TCID_{50}$/ml) with and without PMA treatment; RT-qPCR without PMA treatment (black column) and RT-qPCR with PMA treatment (gray column). x (horizontal) axis stands for different time points (min) applied at 80 °C, and y (vertical) axis stands for virus titers (PCR-units) detected by RT-qPCR assay and calculated to $\log_{10}$ (PCR-units).

3.3.6 RNase treatment to monitor thermal inactivation of HRV stock A

The potential of RNase treatment to discriminate infectious and thermally-treated HRV stock A was evaluated (Figure 9). The titers of both infectious and thermally-treated HRV measured by RT-qPCR assay with RNase treatment (gray column in Figure 9) were significantly ($P < 0.05$) different from the titers measured by RT-qPCR assay alone (black column in Figure 9). After RNase treatment, the titer of infectious HRV was decreased by 0.81 $\log_{10}$ PCR-unit. The reduction level was similar after 1 min of heat treatment, whereas the reduction level was increased by 2.6 $\log_{10}$ PCR-units after 5 min and slightly more after 10 min, and maximum decrease (2.9 $\log_{10}$ PCR-units) was obtained after 60 min of heat treatment.

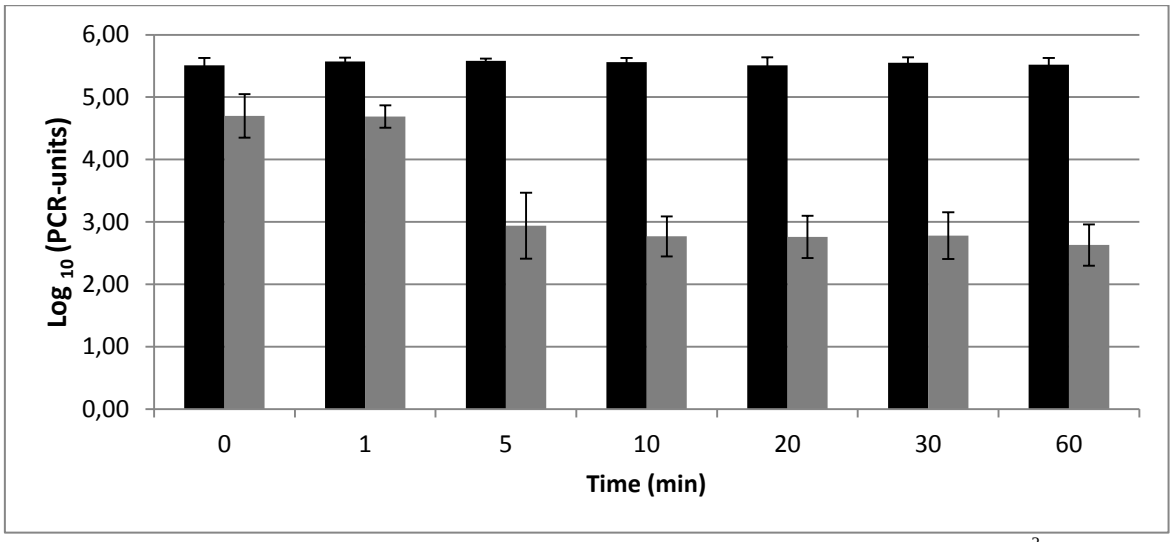

Figure 9. Quantification of infectious and thermally-treated HRV stock A (containing 3.29 x 10^3 $TCID_{50}$/ml) with and without RNase treatment; RT-qPCR without RNase treatment (black column) and RT-qPCR with RNase treatment (gray column). x (horizontal) axis stands for different time points (min) applied at 80 °C, and y (vertical) axis stands for virus titers (PCR-units) detected by RT-qPCR assay and calculated to log_{10} (PCR-units).

3.3.7 Comparison of PMA and RNase treatment on HRV stock A

The efficiencies of PMA and RNase treatments were compared for their potential to discriminate infectious and thermally-inactivated HRV for 60 min at 80 °C, which treatment completely destroyed viral infectivity according to the infectivity assay (Table 10). PMA (100 µM) treatment of inactivated HRV stock A decreased the virus titer by 1 log_{10} PCR-unit, whereas RNase (0.08 mg) treatment reduced the titer by 2.89 log_{10} PCR-units. The higher reduction level of inactivated HRV for 60 min at 80 °C was observed by RNase treatment than PMA treatment.

Table 10. Comparison of PMA and RNase treatment between infectious and inactivated HRV stock A at 80 °C for 60 min.

		PMA		**RNase**	
	PMA or RNase	Quantification [a] Log_{10} (PCR-units)	Reduction	Quantification [a] Log_{10} (PCR-units)	Reduction
Infectious HRV	-	5,68 ± 0,25		5,51 ± 0,12	
	+	4,39 ± 0,05	**1,29**[b]	4,70 ± 0,35	**0,81**[b]
Inactivated HRV (60 min at 80 °C)	-	5,63 ± 0,28		5,52 ± 0,11	
	+	4,57 ± 0,21	**1,07**[c]	2.63 ± 0,33	**2,89**[c]

a. HRV titers (PCR-units) were calculated by plotting the samples against the standard curve, and standard deviations were calculated among the biological replicates.
b. Reduction in quantification between infectious virus before and after PMA or RNase treatment.
c. Reduction in quantification between inactivated virus before and after PMA or RNase treatment.

3.3.8 PMA treatment to monitor thermal inactivation of HRV stock B

The potential of PMA treatment to HRV stock B was also evaluated (Figure 10). First, using RT-qPCR assay alone, the titers of both infectious and thermally-treated HRV stock B (black column in Figure 10) were significantly ($P < 0.05$) different from the titers of those HRV stock A (black column in Figure 8). In comparison to the titer of infectious HRV stock A, the titer of infectious HRV stock B was decreased by 1 $\log_{10}$ PCR-unit. The titers of thermally-treated HRV stock B were gradually decreasing from 1 min to 10 min, and remained constant until 60 min of heat treatment. Moreover, the titers of both infectious and thermally-treated HRV stock B measured by RT-qPCR assay with PMA treatment (gray column in Figure 10) were significantly different ($P < 0.05$) from the titers of those HRV stock B measured by RT-qPCR assay alone (black column in Figure 10). After PMA treatment, the titer of HRV stock B without heat treatment reduced by 1.77 $\log_{10}$ PCR-units, while the reduction level was not higher for thermally-treated HRV stock B; it gradually decreased from 1 min to 10 min, and it rendered similar (less than 1 $\log_{10}$ PCR-unit) for the viral samples inactivated more than 10 min of heat treatment.

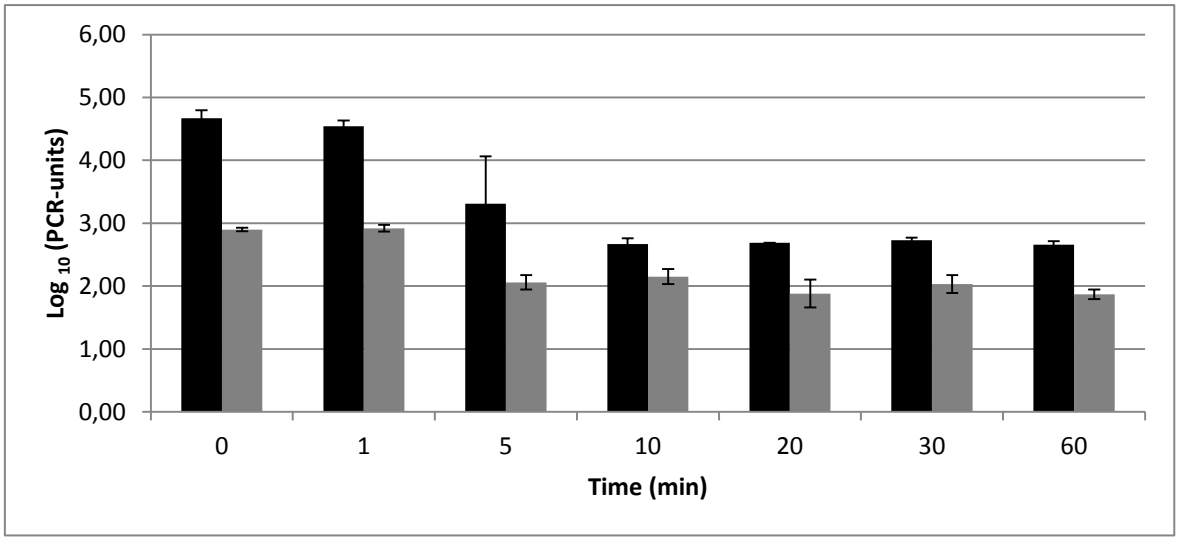

Figure 10. Quantification of infectious and thermally-treated HRV stock B with and without PMA treatment; RT-qPCR without PMA treatment (black column) and RT-qPCR with PMA treatment (gray column). x (horizontal) axis stands for different time points (min) applied at 80 °C, and y (vertical) axis stands for virus titers (PCR-units) detected by RT-qPCR assay and calculated to $\log_{10}$ (PCR-units).

3.3.9 RNase treatment to monitor thermal inactivation of HRV stock B

The potential of RNase treatment to HRV stock B was also evaluated (Figure 11). The titers of both infectious and thermally-treated HRV stock B measured by RT-qPCR assay with RNase treatment (gray column in Figure 11) were not significantly ($P > 0.05$) different from the titers of those HRV stock B measured by RT-qPCR assay alone (black

column in Figure 11). In contrast to the previous result, the effect of RNase treatment to HRV stock B was negligible as it decreased the titers of both infectious and thermally-treated HRV by less than 1 log_{10} PCR-unit.

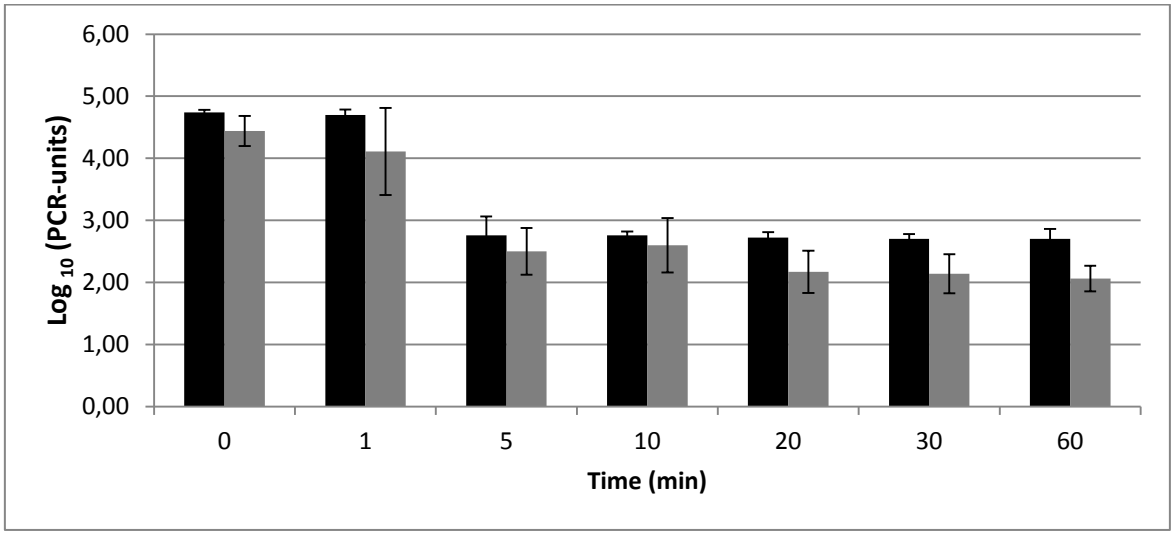

Figure 11. Quantification of infectious and thermally-treated HRV stock B with and without RNase treatment; RT-qPCR without RNase treatment (black column) and RT-qPCR with RNase treatment (gray column). x (horizontal) axis stands for different time points (min) applied at 80 °C, and y (vertical) axis stands for virus titers (PCR-units) detected by RT-qPCR assay and calculated to log_{10} (PCR-units).

3.3.10 Comparison of PMA and RNase treatment on HRV stock B

The efficiencies of these two treatments were also compared for their potential to discriminate infectious and thermally-inactivated HRV stock B for 60 min at 80 °C (Table 11). PMA (100 µM) treatment reduced the titer of infectious HRV stock B by almost 2 log_{10} PCR-units, whereas less than 1 log_{10} PCR-unit reduction was obtained for thermally-inactivated HRV stock B. RNase (0.08 mg) treatment decreased the titers of both infectious and inactivated HRV stock B by less than 1 log_{10} PCR-unit. Both treatments decreased the titers of both infectious and inactivated virus by less than 1 log_{10} PCR-unit.

Table 11. Comparison of PMA and RNase treatment between infectious and inactivated HRV stock B at 80 °C for 60 min.

		PMA		**RNase**	
	PMA or RNase	Quantification [a] Log_{10} (PCR-units)	Reduction	Quantification [a] Log_{10} (PCR-units)	Reduction
Infectious HRV	-	4,67 ± 0,13		4,74 ± 0,04	
	+	2.90 ± 0,03	**1,77**[b]	4,44 ± 0,24	**0,30**[b]
Inactivated HRV (60 min at 80 °C)	-	2,66 ± 0,05		2,70 ± 0,16	
	+	1.87 ± 0.08	**0,79**[c]	2.06 ± 0.21	**0,64**[c]

a. HRV titers (PCR-units) were calculated by plotting the samples against the standard curve, and standard deviations were calculated among the biological replicates.
b. Reduction in quantification between infectious virus before and after PMA or RNase treatment.
c. Reduction in quantification between inactivated virus before and after PMA or RNase treatment.

3.4 Discussion

Despite the significance of rotavirus in public health, current methods for rotavirus detection are limited mainly due to the lack of information on viral infectivity which is critical to assess infectious risk to population (Gassilound and others 2003; Duizer and others 2004; Choi and Jian 2005; Rodriguez-Lazaro and others 2012). In order to establish a reliable and reproducible molecular method for rotavirus detection with information of viral infectivity, the potential of using RT-qPCR assay combined with PMA or RNase treatment to measure the infectivity of human rotavirus (HRV) was investigated.

The validation of PMA and RNase treatments

In order to assess the viral infectivity, several approaches have recently been developed on the basis of using PCR assays. First, one of the promising approaches is pre-treatment of virus sample with a viability dye such as ethidium monoazide (EMA) or propidium monoazide (PMA), prior to PCR-based assay (Fittipaldi and others 2011). Both EMA and PMA are DNA intercalating dyes that can possibly only penetrate to the damaged or compromised capsid of inactivated or non-infectious viruses, and covalently bind to viral RNA upon light exposure, and inhibit its amplification by PCR assay (Gensberger and Kostic 2013). On the other hand, the dyes possibly do not penetrate to intact capsid of infectious viruses, and thus those viral RNA of infectious viruses can successfully amplify by PCR assay (Fittipaldi and others 2012). Due to the lack of specificity of EMA treatment for intact cells and higher permeability of PMA than EMA to intact cells of bacteria, PMA treatment seems to be more promising. Some studies have evaluated the applicability of PMA treatment combined with PCR-based assays on enteric viruses; bacteriophage T4 (Fittipaldi and others 2010), poliovirus (Parshionikar and others 2010), hepatitis A virus (Sanchez and others 2012), murine norovirus (Kim and others 2012) and recently human rotavirus (Coundray-Meunier and others 2013). Most studies claimed that PMA treatment combined with PCR-based assay would be a useful tool to assess the viral infectivity under defined condition, as the applicability of this assay was depending on virus species and inactivation methods applied. The generation of false-positive signals remains to be the greatest concern.

In this study, the effects of PMA treatment on infectious and thermally-treated HRV stock A were preliminarily tested. First, the light exposure and PMA itself without light exposure had no effect on infectious virus. On the other hand, PMA treatment (50 and 100 µM) upon light exposure decreased the titer of infectious virus slightly more than 1 $\log_{10}$ unit

(reduction levels from 1.16 to 1.29 $\log_{10}$ units). The similar reduction levels (from 0.09 to 1.21 $\log_{10}$ units) were observed when PMA was treated to thermally-treated viruses regardless of PMA concentrations and temperatures used for inactivation. These results were in disagreement with the ones from previous studies; the addition of PMA to infectious virus did not have strong effects on virus titer detected by PCR assays, whereas higher reduction level of virus titer were obtained when PMA was treated to inactivated viruses. For instance, Coundray-Meunier and others (2013) found that PMA treatment to infectious rotavirus decreased the virus titer ranging from 0.05 $\log_{10}$ to 0.63 $\log_{10}$ units depending on PMA concentration used (50, 75 and 100 µM), whereas they observed the maximum decrease (about 1.45 $\log_{10}$ units) for thermally-inactivated rotavirus (for 10 min at 80 °C) with PMA at a final concentration of 100 µM. Fittipaldi and others (2010) observed almost 7 $\log_{10}$ units reduction for thermally-inactivated bacteriophage T4 viruses (for 15 min at 110 °C) using PMA treatment (100 µM) whereas only 0.66 $\log_{10}$ unit reduction for infectious virus. In addition, Sanchez and others (2012) observed that PMA treatment to infectious hepatitis A virus reduced its titer less than 0.35 $\log_{10}$ unit, while more than 2.5 $\log_{10}$ unit reduction was obtained with thermally-inactivated viruses (for 5 min at 99 °C). Thus, the validity of PMA treatment on our HRV stock A was doubtful due to similar reduction levels observed between infectious and inactivated viruses.

Accordingly, it was hypothesized that PMA concentration chosen for this study may not be sufficient enough to bind to all of viral RNA in HRV stock A, so that the binding of PMA to viral RNA was tested. As PMA treatment of 100 µM to thermally-treated viruses yielded a slightly higher reduction level (0.03 $\log_{10}$ units) than 50 µM, PMA at a final concentration of 100 µM was chosen for this experiment. In contrast to our hypothesis, 4 $\log_{10}$ units reduction was obtained with PMA treatment to the given amount of viral RNA (1.0×10^6 PCR-units) upon light exposure. The reduction level of 3 $\log_{10}$ units was observed after RNA purification, implying the inhibitory effect of unbound PMA or excess PMA. The reduction levels in our study were even higher than the ones in the previous study of Coundray-Meunier and others (2013) where the reduction level of 2 $\log_{10}$ units was observed when 10^8 genome copies of viral RNA were treated with PMA at concentration of 100 µM. Our result indicated that PMA at a final concentration at 100 µM was able to sufficiently bind to all of viral RNA. In addition, another minor finding in this experiment was that PMA (100 µM) had inhibitory effect on viral RNA without light exposure, as only 0.38 $\log_{10}$ PCR-unit was detectable which was below the detection limit of RT-qPCR assay. However, the inhibitory effect was efficiently removed by RNA

purification step. This finding corresponded to the study where PMA concentrations ranging from 50 to 200 µM totally inhibited RT-qPCR amplification of viral RNA (Coundray-Meunier and others 2013). In consequence, PMA at final concentration of 100 µM was chosen for further studies in order to investigate its potential to assess the viral infectivity in comparison to RT-qPCR assay alone and cell culture-based infectivity assay.

Next, another promising approach to assess the viral infectivity is enzymatic treatment combined with RT-qPCR assay. This approach was first introduced by Nuanualsuwan and Cliver (2002). They used the pre-treatment of virus samples with Proteinase K and RNase prior to RT-PCR assays. Based on the fact that the viral infectivity requires the functional integrity of viral capsid and viral genome, they added Proteinase K to degrade capsid proteins damaged by inactivation, and then RNase to reach viral RNAs released from the degraded viral capsids. In their study, hepatitis A virus, poliovirus, and feline calicivirus were thermally inactivated at 72 °C, and both enzymes were treated prior to RT-PCR. Enzymes treated viruses gave negative PCR results, while positive result was obtained with PCR without any enzymes digestion. Later, some studies have investigated the applicability of enzymatic treatment combined with PCR-based assays on enteric viruses; bacteriophage MS2 (Pecson and others 2009), hepatitis A virus (Sanchez and others 2012), human norovirus (Rönnqvist and others 2014), and murine norovirus (Baert and others 2008; Rönnqvist and others 2014). Most studies suggested that this approach could be useful to track infectivity at some degree, while the applicability of this assay may be limited due to the generation of false-positive results.

In this study, the effects of RNase treatment on infectious and thermally-treated HRV stock A were preliminarily tested. Although the use of both Proteinase and RNase had synergistic effect on signal reduction (Baert and others 2008; Rönnqvist and others 2013), RNase treatment alone was used in this study. This was because the purpose of using Proteinase in previous studies was mainly to attack the viral capsids, so that high concentration of Proteinase K may also affect intact capsids. Thus, the absence of Proteinase K was expected to be negligible. RNase treatment (0.04 and 0.08 mg) decreased the titer of infectious virus by slightly more than 1 $\log_{10}$ unit (reduction levels from 1.04 to 1.26 $\log_{10}$ units). This reduction level was slightly higher than previous studies; Baert and others (2008) found that the use of RNase treatment alone did not have strong impact on the number of genome copies of the unheated murine noroviruses, and Rönnqvist and others (2013) observed that the loss of RNA was less than one log unit after the enzymatic

treatment of infectious viruses. The reason for obtaining higher reduction level in this study may imply the presence of external viral RNA in our virus stock.

Moreover, the similar reduction level (about 1 $\log_{10}$ unit reduction) was observed for the viruses incubated at 37 °C for 30 min. On the other hand, maximum decrease (3 $\log_{10}$ unit reduction) was obtained for thermally-treated virus at 80 °C and 97 °C for 30 min. Similar results were found in previous studies; Nuanualsuwan and Cliver (2003) found that capsid of those inactivated viruses at 37 °C were still detectable after RNase treatment, while that of those inactivated at 72 °C was no longer detectable. Since the thermal inactivation at 72 °C significantly attacks the viral capsids, the capsids became susceptible to both enzymes and can no longer protect viral RNA. Moreover, Pecson and others (2009) observed that the elimination of false-positive signals using enzymatic treatment differed between inactivating treatments. Accordingly, this corresponding result may demonstrate the limitation of using this method in that capsid integrity as criterion for viral infectivity may be limited, while at the same time, it indicated that RNase treatment may be able to attack RNA in case of thermal inactivation at 80 °C.

Even though the similar reduction level was observed for both RNase doses, 0.08 mg of RNase was chosen for further studies to ensure sufficient amount of RNase to degrade the external RNA released from damaged virus particles. This was based on the previous study by Rönnqvist and others (2013) where they observed that too high concentration of proteinase reduced the number of presumably viable viruses whereas too low concentration of either enzyme was not enough to digest the damaged virus particles. They suggested the importance of enzyme concentration and balance between proteinase and RNase enzyme.

PMA and RNase treatments to monitor thermal inactivation of HRV stock A

From preliminary test, heat treatment at 80 °C as one of disinfection methods was selected as it was expected to cause the damage on viral capsid, and different time points (0, 1, 5, 10, 20, 30, and 60 min) were employed to investigate the potential of PMA and RNase treatment to discriminate between infectious and thermally-treated rotavirus. First, thermal inactivation curve of original HRV stock was generated using the cell culture-based infectivity assay. After 1 min of heat exposure at 80 °C, the virus titer was dramatically decreased from 3, 29 x 10^5 $TCID_{50}$/ml to 1, 04 x 10 $TCID_{50}$/ml (4 $\log_{10}$ reduction). After 5 min, infectivity was completely destroyed (> 5 $\log_{10}$ reduction). It demonstrated that the heat exposure at 80 °C for 5 min was sufficient to inactivate the rotavirus. In previous study, Ojeh and others (1995) found that autoclaving for 15 min at 121°C completely

destroyed the infectivity of rotavirus as well as amplifiable viral RNA by PCR assay. Moreover, Estes and others (1979) found that heating at 50 °C for 30 min inactivates 99% of the rotavirus infectivity. Mahony and others (2000) observed more than 7 $\log_{10}$ reduction of rotavirus infectivity after the heat exposure at 60 °C 10 min.

Next, the RT-qPCR assay with and without pre-treatments were applied to measure the titers of HRV stock A before and after heat treatment. Due to the higher sensitivity of RT-qPCR assay than cell culture-based infectivity assay, HRV stock A was prepared by diluting to 10^{-2} of original HRV stock. Using RT-qPCR assay without any pre-treatments, the titer of infectious virus was about 5.5 $\log_{10}$ units, while the titer of thermally-treated virus remained constant even after heat treatment for 60 min at 80 °C. Although the results between cell culture (original HRV stock) and RT-qPCR assay (HRV stock A) were not equivalently comparable in this study due to different concentrations of HRV stocks, the discrepancy of results between cell culture and RT-qPCR assays demonstrated that RT-qPCR assay without any pretreatment overestimated the infectivity of thermally-treated viruses. Similar result was observed in previous study by Baert and others (2008) where found that after heat exposure for 1 hour at 80 °C, more than 6 $\log_{10}$ genomic copies of murine norovirus was still detected by qPCR assay whereas more than 6 $\log_{10}$ unit reduction was observed using plaque assay only after 150 s at 80 °C. This result can be partially explained by the mechanism of heat inactivation. Volkin and others (1997) suggested that the mechanism of thermal inactivation above 65 °C might cause large irreversible structural change due to extensive protein unfolding. Nuanualsuwan and Cliver (2003) observed that heat treatment at 72 °C caused the conformational change in viral protein of human picornavirus and feline calicivirus, and enormously diminished the functions of capsid. They suggested that the target of thermal inactivation seemed to be viral capsid although it was temperature-dependent. In addition, Gassillound and others (2003) found a great difference in heat resistance between infectious viruses and viral genomes in mineral water. Higher temperature could modify the viral protein capsid, leading to inactivation while it had little effect on the viral genome, and the infectious particles were degraded more rapidly than viral genomes. Baert and others (2008) also claimed that heat inactivation seemed to have a much stronger detrimental effect on viral infectivity than on the integrity of viral genome.

Moreover, several studies found the inconsistency between the number of infectious virus and the number of viral genome detected by PCR assays, leading to the overestimation of viral infectivity and possible viral risk (Gassilloud and others 2003; Duizer and others

2004; Choi and Jian 2005; Bosch and others 2008; Yeh and others 2009; Rodriguez-Lazaro and others 2012). These studies argued a need of interpretation of positive PCR results with caution, and claimed that detection of viral genomes may be necessary but not sufficient for assessment of the infectious risk for human population.

Using RT-qPCR assay with PMA treatment, the titers of thermally-treated viruses (ranges from 4.52 to 4.67 $\log_{10}$ units) were similar as the titer of infectious viruses (4.39 $\log_{10}$ units) regardless of inactivation time. Although PMA treatment decreased the titers of both infectious and thermally-treated viruses by 1 $\log_{10}$ units compared to one without any pre-treatment, the results were in disagreement with the one from cell culture assay. It may imply that PMA (100 μM) did not efficiently bind to inactivated viral particles after heat treatment, although the concentration of 100 μM seemed enough to bind the viral RNA in the preliminary test.

In previous studies, the applicability of PMA treatment to assess the viral infectivity was shown to be depending on inactivation methods as well as virus species. Fittipaldi and others (2010) found that PMA could clearly differentiate non-infectious bacteriophage T4 viruses from infectious viruses in case of extreme damage to viral capsid such as 110 °C of heat treatment but not in case of moderate heating (85 °C). They claimed that disinfection methods which did not directly damage viral capsid might lead to loss of infectivity, but render its capsid uncompromised, and therefore PMA failed to reach viral RNA. Likewise, Parshionikar and others (2010) found PMA treatment did discriminate non-infectious viruses when hypochlorite treatment, heat treatment at 37 °C and 72 °C were applied, but not for heat treatment at 19 °C. They highlighted that PMA can be used to assess the infectivity level under the conditions defined. Moreover, Coundray-Meunier and others (2013) found that the quantity of rotavirus remained constant in heat treatment at 37 °C, although the genome titers of rotavirus following heat treatment at 68 °C to 80 °C became similar to infectious titers when PMA treatment was used. In addition, Kim and others (2012) observed that PMA treatment could distinguish between infectious and thermally-inactivated bacteriophage MS2, but not with murine norovirus. They claimed that the reason for difference in results may be due to difference in the size and secondary structure of nucleic acid of target virus, since the secondary structure of the nucleic acid can affect the binding affinity of PMA (Parshionikar and others 2010).

However, in our case, PMA treatment showed similar levels of reduction (ranges from 1.04 to 1.29 $\log_{10}$ PCR-units) for both infectious and thermally-treated viruses regardless of

temperatures and inactivation time used. Coundray-Meunier and others (2013) proved the potential of using PMA treatment to discriminate between infectious and thermally-inactivated human rotaviruses at 80 °C, indicating that thermal inactivation at 80 °C was supposed to damage the capsids of human rotaviruses. Accordingly, the content of virus stock was thought not to be optimal for this study. Although virus stock was newly produced for this study, our rotavirus strain had been stored for more than 20 years. In consequence, one reason may be that several freezing-thawing during experiments might have easily caused the damage on viral capsid of our stock and had led to release viral RNA. In addition, it might have been that the presence of dead cells (MA-104 cells) which were also collected during production of our virus stock might have disrupted the binding of PMA on our virus stock. Some studies with bacteria have suggested that the presence of high number of dead cells seems to influence the false-positive results of PMA treatment (Wang and others 2009; Fittipaldi and others 2012). Although the reason for influence of dead cells is not currently clear, it may be that high numbers of dead cells might take up the dye resulting in the lower concentration of available dyes, and it is likely that the effectiveness of PMA may be reduced by increasing cell number (Varma and others 2009; Fittipaldi and others 2012).

On the other hand, the results of using RT-qPCR assay with RNase treatment were different from the one with PMA treatment. The titer of heat-treated viruses for 1 min (4.69 $\log_{10}$ units) was similar as the titer of infectious virus (4.70 $\log_{10}$ units) after RNase treatment. Then, the titer of thermally-treated viruses for 5 min was decreased to 2.94 $\log_{10}$ units, and the titer of 2.63 $\log_{10}$ units was observed for thermally-treated virus for 60 min (maximum decrease of 2.9 $\log_{10}$ units). Although the virus titers were gradually decreasing as longer inactivation time applied, this result was not completely in agreement with the one of cell culture assay. While the infectivity of virus after 1 min of heat treatment was reduced about 4 $\log_{10}$ $TCID_{50}$ units in cell culture assay, the titer rendered similar with RNase treatment. It indicated that RNase treatment was not able to degrade the viral RNA in this condition. In consequence, it was hypothesized that 1 min of heat treatment might not be sufficient enough to damage the viral capsid and sufficiently expose RNA, although this condition caused the loss of viral infectivity to some extent. Although it never completely eliminated false-positive results of thermally-treated viruses even after 60 min of heat treatment, RNase treatment did decrease false-positive results of thermally-treated viruses to some extent, and reduction level was correlated to some degree to viral inactivation observed in cell culture assay.

Similar results were found in previous studies. Baert and others (2008) observed the reduction of heat-treated norovirus titer using enzymatic treatment, but high number of genome copies (more than 6 $\log_{10}$ units) was still detected even after enzyme treatment in the absence of infectious virus. Pecson and others (2009) also found that enzymatic treatment reduced the inactivated bacteriophage MS2 titer (more than 5 $\log_{10}$ units) but did not completely eliminate the false-positive signals. They considered this phenomenon as plateau effect, and they suggested that this incomplete degradation (plateau effect) may be related to both the secondary structure of genome and its association with the capsid, which may result in less RNA degradation due to the decreased access between the RNase and the genome. The plateau effect was also observed by Rönnqvist and others (2013). They found that enzymatic treatment reduced false-positive signals of damaged norovirus particles but it did not reach a level at which all inactivated virus particles would have been enzymatically digested. Thus, in accordance with the previous findings, the result in this study indicated that RNase could degrade the inactivated viral particles to some extent, although it could not completely exclude the inactivated viruses.

So far, the RNase treatment seemed to be more effective than PMA treatment to discriminate the infectious and thermally-inactivated HRV stock A in defined condition. This was because thermally-inactivated HRV titer (for 60 min at 80 °C) was decreased by almost 3 $\log_{10}$ PCR-units with RNase treatment, and only 1 $\log_{10}$ PCR-units with PMA treatment. In case of Hepatitis A viruses, Sanchez and others (2012) found that PMA treatment was more effective than RNase treatment for differentiating infectious and thermally-inactivated hepatitis A viruses (for 5 min at 99 °C), as the virus titer reduced by more than 2.4 $\log_{10}$ units with PMA treatment whereas only 0.55 $\log_{10}$ units with RNase treatment. These finding may suggest that the applicability of both pre-treatments may depend on virus species.

PMA and RNase treatments to monitor thermal inactivation of HRV stock B

Yet, in order to confirm the effects of PMA and RNase treatments, further studies were necessary. This was mainly because the result of using RT-qPCR with PMA treatment raised the question whether our HRV stock was optimal for this study. In consequence, HRV stock B was prepared by treating RNase to the HRV stock A in order to remove the possible external RNA or dead cells. The RT-qPCR assay with and without pre-treatments were applied to measure the titers of HRV stock B before and after heat treatment. Using RT-qPCR assay without any pre-treatment, the titer of non-heat treated HRV stock B (4.67 $\log_{10}$ units) was decreased about 1 $\log_{10}$ unit compared to the titer of non-heat treated HRV

stock A (5.65 $\log_{10}$ units). It implied the presence of external RNA outside intact capsid in the original virus stock as expected, and thus only complete viral particles which ideally contained intact capsid protecting viral genome (also called virion), were expected to remain in the HRV stock B. Due to the limitation of PCR assays previously described, it was assumed that the titers of HRV stock B with or without thermal inactivation would be similar using RT-qPCR assay without any pre-treatment. However, the titer of HRV stock B was gradually decreased from 1 min to 10 min of heat treatment, and remained constant after 10 min. This result can be explained by the co-occurrences of two possible hypotheses. First, although RNase was added on the purpose of removing the free external RNA, it was possible that the viral capsids became more vulnerable after RNase treatment with subsequent heat treatment, and in consequence the viral genome might have been released. At the same time, it might be that RNase inhibitor did not properly inhibit the activity of the RNase so that they were still able to bind the released viral genome during heat treatment, although enzyme was supposed to be inactivated after few minutes in 80 °C. These hypotheses could be partially supported by a previous study. Gassillound and others (2003) suggested the slight decrease of viral genome quantities using RT-PCR assay alone after 140 days at 35 °C could have been due to the possible presence of RNase in which might have originated from the environmental or stool samples where virus stock was isolated, and the protection of capsid might have been partially disappeared with time at 35 °C, and in consequence RNase were able to digest viral RNA as soon as it became available outside the capsid. Thus, if these hypotheses above would have been correct, RT-qPCR assay alone might have been discriminating between the infectious and thermally-treated viruses to some extent, and would lead to difficulties in interpreting the true effect of PMA and RNase treatment in further studies.

Using RT-qPCR with PMA treatment, the titer of HRV stock B without heat treatment was reduced by 1.77 $\log_{10}$ units after PMA treatment only. If these hypotheses above would have been accepted, PMA might have also excluded RNA from more vulnerable capsids of the infectious virus synergistically with the remained activity of RNase. Otherwise, it could have been that HRV stock B became non-infectious after RNase treatment, although it could not be confirmed as the infectivity titer of HRV stock B was not measured. On the other hand, the reduction levels of heat-treated HRV stock B (range from 0.52 to 1.62 $\log_{10}$ units) were lower than those of non-heat treated (1.77 $\log_{10}$ units): the titer of heat-treated HRV stock B was gradually decreased from 1 to 10 min, and rendered similar (less than 1 $\log_{10}$ unit) after 10 min of heat treatment. It might have been that PMA could not yield any

higher reduction level after longer time of heat treatment, as it was hypothesized that RT-qPCR assay alone would have been already discriminating between the infectious and thermally-treated viruses to some extent.

In contrast to the previous result of RT-qPCR assay with RNase treatment on HRV stock A, the effect of RNase treatment on HRV stock B was negligible. RT-qPCR assay with RNase treatment decreased the virus titers by less than 1 $\log_{10}$ unit for both HRV stocks B with and without heat treatment. The results of statistical analysis showed that the titers of HRV stock B measured by RT-qPCR assay with or without RNase treatment were significantly correlated. Although RNase treatment was applied twice before and after heat treatment in HRV stock B, it was likely that RNase treatment had the plateau effect due to the decreased access between the RNase and the genome as discussed before. Nevertheless, due to the lack of information on infectivity assay of HRV stock B, the true effect of PMA and RNase treatment to assess the viral infectivity is hard to compare in this condition.

Overall evaluation of PMA and RNase treatments to human rotavirus

All in all, PMA treatment seemed to bind rotavirus RNA to some extent by decreasing the false-positive results, although the high numbers of false-positive signals from thermally-inactivated viruses were still detected even with PMA treatment. One of the main reasons for false-positive results in this study might be the shortness of the target gene region (84 bp) amplified. In previous studies, Coundray-Meunier and others (2013) observed that the reduction level of virus titer was different depending on the RT-qPCR assays where different lengths of target gene were amplified. Moreover, Fittipaldi and others (2012) suggested that the amplification of longer target gene sequence correlates with a higher probability of discriminating between damaged and non-damaged gene, resulting in a stronger suppression of signals from dead cells.

Indeed, the generation of false-positive signals due to incomplete signal suppression has been considered as the greatest concern using this assay, and in consequence, the need of optimizing the PMA treatment has been suggested for a successful application (Fittipaldi and others 2010). Fittipaldi and others (2012) claimed the several important factors to optimize this method; dye concentration, the incubation condition, the light source, the presence of high number of dead cells, the salt concentration or pH of the reaction mix, the length of target gene, and the sequence of the target gene etc. In this study, although RNase treatment was performed to remove the presence of external RNA or dead cells in our virus

stock, this treatment was still unsuccessful to obtain the optimal virus stock. Thus, further studies may be necessary to focus on optimizing the assays such as using different primers, and producing the optimal virus stock in order to extensively evaluate the potential of PMA treatment to assess the infectivity of rotavirus. In addition, studies on the functional changes of rotavirus capsid and genome upon thermal inactivation at 80 °C would be helpful in understanding the effect of PMA on rotavirus.

To our knowledge, this is the first study where RNase treatment was employed to assess the infectivity of human rotavirus. Although RNase treatment seemed to discriminate between infectious and thermally-treated HRV stock A in the early stage of the experiments, the results using HRV stock B gave conflicting findings. Thus it was not possible to confirm its effects on HRV. Nevertheless, RNase treatment seemed to degrade rotavirus RNA to some extent by eliminating the false-positive results of thermally-inactivated rotaviruses, although the discrepancy between infectivity assays and enzymatic treatment with PCR assays was observed. Pecson and others (2009) claimed that the main advantage of using this assays is that it caused a significant decrease of false-positive signals and never contributed an additional false-positive signals, so that this assay would be a valuable tool regarding viral infectivity as the degree of reduction responded to inactivation in manner proportional to cell culture-based assays. In addition, they suggested that the relationship of inactivation curve between the cell culture and enzymatic treatment with PCR assays would need to be established for each virus and inactivating treatment, for the practical application of this assay.

In order to confirm the applicability of RNase treatment to human rotavirus, further studies are necessary to produce the optimal virus stock and inactivation curve for that virus stock. The moderate heating (for 1 min for 80 °C) was not enough to cause the damage on viral capsids. Therefore, attempt to use proteinase prior to RNase treatment as most of other studies would be necessary. Moreover, it would be worth trying to investigate the potential of this assay to discriminate between infectious and inactivated rotavirus after other inactivation methods such as hypochlorite or ultraviolet light that are commonly used in disinfection treatments for drinking water.

4 CONCLUSION

The purpose of the present study was to develop a robust molecular method for rotavirus detection with information of viral infectivity, and which may also contribute to the development of molecular methods for correct estimation of infectivity of non-cultivable health-significant viruses. First, the significance of rotavirus in public health and the need of reliable molecular methods for virus detection and promising methods were reviewed. Second, the potential of using PCR-based molecular assay combined with dye or enzymatic treatments to assess the infectivity of human rotavirus were evaluated.

In summary, this study demonstrates that heat exposure at 80 °C for 5 min was sufficient to inactivate human rotavirus based on the infectivity assay, and shows that RT-qPCR assay did not distinguish between infectious and inactivated viral genomes, resulting in the overestimation of viral infectivity. Moreover, this study shows that RT-qPCR assay combined with PMA and RNase pre-treatment eliminated the false-positive results of RT-q PCR assay to some extent in defined conditions. However, this study still finds discrepancy between the infectivity assay and RT-qPCR assays even with PMA or RNase treatment. The current investigation was limited by the use of possibly non-optimal HRV stock.

Thus, in order to confirm the potential of using PCR-based assay combined with PMA or RNase treatment to measure the infectivity of human rotavirus, further studies on optimization of PMA and RNase treatment as well as production of the optimal HRV stock would be necessary. Further studies on the functional changes of capsid and genomes of HRV by thermal inactivation would help to evaluate the effects of PMA and RNase treatments on thermally treated human rotavirus. Nevertheless, this study confirms the previous findings and contributes additional evidence that suggests the potential of using these assays to assess the infectivity of non-cultivable viruses in defined condition.

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