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Methods to detect infectious human enteric viruses in environmental water samples

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ABSTRACT

Currently, a wide range of analytical methods is available for virus detection in environmental water samples. Molecular methods such as polymerase chain reaction (PCR) and quantitative real time PCR (qPCR) have the highest sensitivity and specificity to investigate virus contamination in water, so they are the most commonly used in environmental virology. Despite great sensitivity of PCR, the main limitation is the lack of the correlation between the detected viral genome and viral infectivity, which limits conclusions regarding the significance for public health. To provide information about the infectivity of the detected viruses, cultivation on animal cell culture is the gold standard. However, cell culture infectivity assays are laborious, time consuming and costly. Also, not all viruses are able to produce cytopathic effect and viruses such as human noroviruses have no available cell line for propagation. In this brief review, we present a summary and critical evaluation of different approaches that have been recently proposed to overcome limitations of the traditional cell culture assay and PCR assay such as integrated cell culture-PCR, detection of genome integrity, detection of capsid integrity, and measurement of oxidative damages on viral capsid protein. Techniques for rapid detection of infectious viruses such as fluorescence microscopy and automated flow cytometry have also been suggested to assess virus infectivity in water samples.

Introduction

Despite the progress in water and wastewater treatment technology, waterborne diseases still have public health and socio-economic implications in both the developed and developing world. According to the World Health Organization (WHO), every year there are ~2.2 million deaths related to unsafe water, sanitation and hygiene, and millions more suffer multiple episodes of non-fatal diarrhoea (WHO, 2008).

Viruses are a major cause of waterborne and water-related diseases. The health impact of these viruses varies; some waterborne viruses cause gastroenteritis, respiratory infections, conjunctivitis, hepatitis, aseptic meningitis, encephalitis and paralysis as shown in Table 1. Moreover, the infective dose can be very low, for instance it has been estimated that ~10–100 virions of norovirus are capable of causing human infection (Lindesmith et al., 2003). Thus, even the presence of a few viral particles poses a threat to public health. Sometimes the role of viruses in waterborne and water-related diseases is difficult to assess. This could be due to a lack of data, difficulties to define the source of infections, sub-clinical infections and secondary transmission, in addition the virus may escape the detection method (Grabow, 2007).

In recent years, a number of waterborne outbreaks of human enteric viruses have been reported as illustrated in Table 2.

Abbreviations: 5′-UTR, five prime untranslated region; A549, human lung carcinoma cells; BGM, buffalo green monkey kidney cells; CFP, cyan fluorescent protein; CPE, cytopathic effect; ESI, electrospray ionization; ELISA, enzyme-linked immunosorbent assay; EMA, ethidium monoazide; EV, enterovirus; FrhK-4, fetal rhesus monkey kidney cells; FC, flow cytometry; FRET, fluorescence resonance energy transfer; FACS, fluorescence-activated cell-sorting; HAdV, human adenovirus; HEp-2, human epithelial type 2 cells; IFA, immunofluorescence assay; IMS, immunomagnetic separation; ICC-PCR, integrated cell culture PCR; ICAM-1, intercellular adhesion molecule-1; JCPyV, JC polyomavirus; LAMP, loop-mediated isothermal amplification; MA104, simian kidney epithelial cells; MS, mass spectrometries; MALDI, matrix assisted laser desorption ionization; MBs, molecular beacons; MPN, most probable number; NASBA, nucleic acid sequence based amplification; PCR, polymerase chain reaction; PLC/PRF/5, primary liver carcinoma cells; PMA, propidium monoazide; qPCR, quantitative real time polymerase chain reaction; RT-PCR, reverse-transcription- polymerase chain reaction; RD, rhabdomyosarcoma cells; CaCo-2, human colon carcinoma cells; T_{99}, time needed to produce a 2-log reduction in the initial concentration; TCID50, 50% tissue culture infectious dose; UV, ultraviolet light; Vero, African green monkey kidney cells; WHO, World Health Organization; YFP, yellow fluorescent protein.

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Table 1
Human viruses that have possible association with waterborne transmission.

Family	Genera	Disease	References
Caliciviridae	Norovirus, Sapovirus	Gastroenteritis	Schwab (2007)
Reoviridae	Rotavirus (A–C) Orthoreovirus (Reovirus)	Gastroenteritis Respiratory tract disease, gastroenteritis	Schwab (2007)
Astroviridae	Astroviruses (human astrovirus)	Gastroenteritis	Schwab (2007)
Adenoviridae	Mastadenovirus (human adenovirus A–F)	Respiratory diseases, pneumonias, keratoconjunctivitis, cystitis, gastroenteritis	Jiang (2006)
Polyomaviridae	Polyomavirus (JC; BK; KI; WU; MC)	Progressive multifocal leukoencephalopathy, kidney nephritis, respiratory and Merkel cell carcinoma is also suspected	Bofill-Mas et al. (2006, 2010); Hamza et al. (2009a)
Parvoviridae	Bocavirus (human bocavirus)	Unclear, gastroenteritis, respiratory infection	Allander et al. (2005); Hamza et al. (2009b)
Hepeviridae	Hepevirus (hepatitis E virus)	Hepatitis	Pintó and Saiz (2007)
Picornaviridae	Hepatovirus (hepatitis A virus)	Hepatitis	Pintó and Saiz (2007)
	Enterovirus (human enterovirus A–D; human rhinovirus A–C); Kubovirus (Aichi virus)	Gastroentiritis, paralysis, meningitis, myocarditis, keratoconjunctivitis, respiratory disease, diabetis	Hovi et al. (2007)
Anelloviridae	Torque teno virus	Unclear, hepatitis	Hamza et al. (2011); Okamoto et al. (1998)
Orthomyxoviridae	Picobirnavirus (human picobirnavirus)	Gastroenteritis	Hamza et al. (2011)
Orthomyxoviridae	Influenza virus	Influenza, respiratory disease	Markwell and Shortridge (1982)
Coronaviridae	Coronavirus	Respiratory, gastroenteritis	Leung et al. (2003)

Table 2
Recently reported waterborne viral outbreaks in different locations. Some of recreational waterborne viral outbreaks adapted from Sinclair et al. (2009).

Year	Location	Source	Viruses	Reference
2000	Australia	Pool	Adenovirus	Harley et al. (2001)
2000	Italy	Pool	Echovirus	Manzara et al. (2002)
2000	Italy	Drinking water	Norovirus	Boccia et al. (2002)
2000	Albania	Drinking water	Rotavirus, astrovirus, adenovirus, and norovirus	Villena et al. (2003)
2001	South Africa	Pool	Echovirus	Yeats et al. (2005)
2001	Germany	Pool	Echovirus	Hauri et al. (2005)
2001	Finland	Pool	Norovirus, Astrovirus	Maunula et al. (2004)
2001	Minneosta	Lake	Norovirus	Yoder et al. (2004)
2001	Wyoming	Drinking water	Norovirus	Anderson et al. (2003)
2002	The Netherlands	Fountain	Norovirus	Hoebe et al. (2004)
2002	Arizona	River	Norovirus	Jones et al. (2009)
2002	Minneosta	Pool	Norovirus	Yoder et al. (2004)
2002	Minneosta	Lake	Norovirus	Yoder et al. (2004)
2002	Wisconsin	Pool	Norovirus	Yoder et al. (2004)
2002	Wisconsin	Lake	Norovirus	Yoder et al. (2004)
2002	Albania	Drinking water	Rotavirus, astrovirus, adenovirus and calicivirus, hepatitis A virus	Divizia et al. (2004)
2003	Connecticut	Pool	Echovirus	Yoder et al. (2004)
2004	Mexico	Ocean	Coxsackievirus, Echovirus	Begier et al. (2008)
2004	Vermont	Pool	Norovirus	Podewils et al. (2007)
2004	Sweden	Lake	Norovirus	Sartorius et al. (2007)
2005	Minnesota	Lake	Norovirus	Yoder et al. (2008)
2005	India	Drinking water	Hepatitis E	Sarguna et al. (2007)
2005	Brazil	Drinking water	Rotavirus	Siqueira et al. (2010)
2005	Iraq	Drinking water	Hepatitis E virus	Al-Nasrawi et al. (2010)
2005	Turkey	Drinking water	Rotavirus A	Koroglu et al. (2011); Yoder et al. (2008)
2006	Wisconsin	Pool	Norovirus	Yoder et al. (2008)
2006	Florida	Lake	Norovirus	Hewitt et al. (2007); Yoder et al. (2008)
2006	New Zealand	Drinking water	Norovirus	Hewitt et al. (2007); Tallon et al. (2008)
2006	North Carolina	Springwater	Hepatitis A	Prinja et al. (2008); Tallon et al. (2008)
2006	India	Drinking water	Hepatitis E	Cao et al. (2009); Prinja et al. (2008)
2006	China	Drinking water	Hepatitis A	Cao et al. (2009); Lee et al. (2008)
2007	Korea	Drinking water	Hepatitis A	Lee et al. (2008); ter Waarbeek et al. (2010)
2007	Belgium	Drinking water	Norovirus	Rasanen et al. (2010); ter Waarbeek et al. (2010)
2007	Finland	Drinking water	Rotavirus, calicivirus, Aichi virus, adenovirus, and bocavirus	Maunula et al. (2009); Rasanen et al. (2010)
2007	Finland	Drinking water	Norovirus, astrovirus, rotavirus, enterovirus and adenovirus	Maunula et al. (2009); Werber et al. (2009)
2008	Montenegro	Drinking water	Norovirus, rotavirus and adenovirus	Scarcella et al. (2009); Werber et al. (2009)
2009	Italy	Drinking water	Norovirus, rotavirus, enterovirus or astrovirus	Riera-Montes et al. (2011); Scarcella et al. (2009)
2009	Sweden	Drinking water	Norovirus	Riera-Montes et al. (2011)

Accordingly, there is a need for rapid and reliable methods to detect small numbers of infectious virus particles in environmental samples. However, the analysis of viruses in environmental water samples is a complex process that can be divided into two main steps: sample concentration and virus detection.

Different methods have been used for the enrichment of viruses from environmental water samples such as adsorption/elution protocols (electronegative membrane, electropositive membrane, electronegative cartridges, electropositive cartridges, glass wool and glass powder), entrapment ultrafilltration (alginate membranes, single membranes and hollow fibres), ultracentrifugation and hydroextraction (Wyn-Jones and Sellwood, 2001).

Currently, a wide range of analytical methods is available for virus detection in environmental water samples such as enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), reverse-transcription PCR (RT-PCR), real-time PCR, real-time RT-PCR, nucleic acid sequence based amplification (NASBA), real time NASBA, loop-mediated isothermal amplification (LAMP) and reverse transcription LAMP; more details are reviewed in Mattison and Bidawid (2009).

Basically cell culture system is the gold standard to examine the infectivity of the isolated viruses. There are some enteric viruses which are easy to propagate (enteroviruses), others are difficult to propagate (rotavirus and hepatitis A virus), and for some there are no cell lines available for propagation (human noroviruses). Although an infectivity assay for norovirus has recently been described, this assay requires highly differentiated 3D cell culture of human embryonic intestinal epithelial cell line INT-407, which can support the natural growth of human NoV (Straub et al., 2007). Since the assay itself requires a cell line that is not widely available and combined with the required special technique, it is difficult to use on a routine basis.

The major drawback of conventional cell culture assays is that they cannot be used as routine and robust detection tools because they are laborious, expensive and conditions under which many important enteric pathogens can be cultured are not known. Therefore, PCR-based methods are commonly used to detect and identify virus contamination in water particularly those viruses which do not multiply in cell culture.

The specificity of the PCR can be confirmed by hybridization of the PCR product to a labelled internal oligonucleotide probe or by sequencing the DNA product or by using nested or semi-nested PCR. Detection of enteric viruses using PCR or RT-PCR has been extensively described for aquatic environments (Mattison and Bidawid, 2009). Additionally, the multiplex PCR can be used for detection of several types, groups or species in one reaction. In real time PCR assays, amplification and detection of viral genome are combined in one reaction leading to faster availability of results and reduction of the risk of false positive results by contamination with PCR products. Although molecular methods have the highest degree of sensitivity and specificity, the co-concentration of PCR inhibitors from environmental water samples may represent a limitation for the use of PCR as a detection method. Therefore, it is important to include controls for detection of PCR inhibition for each sample. The inhibitory effect can then be relieved or reduced by several approaches such as pre-dilution of the extracted nucleic acid prior to PCR reaction (Brooks et al., 2005; Hamza et al., 2009a), the use of some PCR additives (Demeke and Adams, 1992; Kreader, 1996), removal of inhibitors during nucleic acid purification (Braid et al., 2003) and the use of some polymeric adsorbents (Abbaszadegan et al., 1993; Koonjul et al., 1999; Schriewer et al., 2011).

Another disadvantage of direct PCR is the ability to detect naked nucleic acids, infectious and non-infectious pathogens. Consequently, direct PCR does not allow for the discrimination between infectious and non-infectious viral particles (Choi and Jiang, 2005; Hamza et al., 2011).

Herein, we give an overview and evaluation of strengths and weaknesses of different approaches (Fig. 1) that have been recently described to overcome limitations of the traditional cell culture assay and PCR assay to provide information about the infectivity of human enteric viruses in environmental water samples.

Cell culture

Although the ultimate confirmation of viral infectivity is by infection of the natural host, viruses can also be considered to be infectious when they can penetrate the cell membrane of a susceptible cell and express at least one viral gene and/or replicate their genome. Examples of well-known cell types that are standard for environmental virology laboratories are buffalo green monkey (BGM) kidney cells, African green monkey kidney (Vero) cells, primary liver carcinoma (PLC/PRF/5) cells, fetal rhesus monkey kidney cells (FrhK-4), human epithelial type 2 (HEp-2) cells, human lung carcinoma cells (A549), rhabdomyosarcoma (RD) cells, human colon carcinoma cells (CaCo-2), and simian kidney epithelial (MA104) cells (Abad et al., 1998; Ali and Abdel-Dayem, 2003; Ali et al., 2004; Bosch et al., 2004; Chapron et al., 2000; Dahling et al., 1974; Dahling and Wright, 1986; Ehlers et al., 2005; El-Senousy et al., 2007; Fout et al., 1996; Frosner et al., 1979; Hashimoto et al., 1991; Ko et al., 2003; Morris, 1985; Pinto et al., 1994; Rodriguez et al., 2008; Sedmak et al., 2005). Combination of more than one cell line may allow the detection of unanticipated agents, rather than focusing on the detection of only one or a few specific viruses. For example a combination of A549 and BGMK cells and molecular identification could be a useful tool for monitoring infectious adenoviruses (AdV) and enteroviruses (EV) in aquatic environments (Lee et al., 2004). Indeed, no single cell line is able to propagate all viruses even within the same virus group. For instance, among the EV group, which includes polioviruses, coxsackieviruses, echoviruses, and other numbered serotypes of EV, there is much similarity in terms of structure and surface antigens, however, there is no single cell line that allows proliferation of all EV strains (Leland and Ginocchio, 2007). If EV is the virus of interest in a water sample, cell lines such as BGM, CaCO-2 or RD can be used. Furthermore, detection of EV serotype spectrum can be broaden by the use of human embryonic lung or kidney cells, primary monkey kidney cells or Vero cells, as well as an intercellular adhesion molecule-1 (ICAM-1) over-expressing HeLa cell line (Hovi et al., 2007).

Infectivity assay is always conducted through adsorption inoculation by decanting the cell culture medium from the cell culture monolayer and applying the inoculum directly to the monolayer. After a 30–90-min incubation of the inoculated tube in a horizontal position at 37 °C, excess inoculum is discarded and fresh maintenance cell culture medium is added (Leland and Ginocchio, 2007). Cytopathogenic or cytopathic effect (CPE) of the virus is monitored by microscopic examination daily for the first week of incubation to maximize the detection of viral growth and on alternate days for the remainder of the incubation period. Some viruses take 1 day to 3 weeks to produce a CPE depending on the initial concentration and virus type. CPE can be swelling, shrinking, rounding of cells to clustering or complete destruction of the monolayer (Leland and Ginocchio, 2007). This can also be done in a quantitative form and the virus can be enumerated as most probable number (MPN) (Chapron et al., 2000; Gantzer et al., 1998; Shieh et al., 2008) or tissue culture infectious dose (TCID50) units (Calgua et al., 2011; Enriquez and Gerba, 1995; Jiang et al., 2004; Kok et al., 1998). MPN test involves observation of CPE in monolayers inoculated with different dilutions of viral suspension and subsequent use of MPN table or MPN software program to determine the viral counts. In TCID50 test the titre is calculated as the logarithm of the dilution of

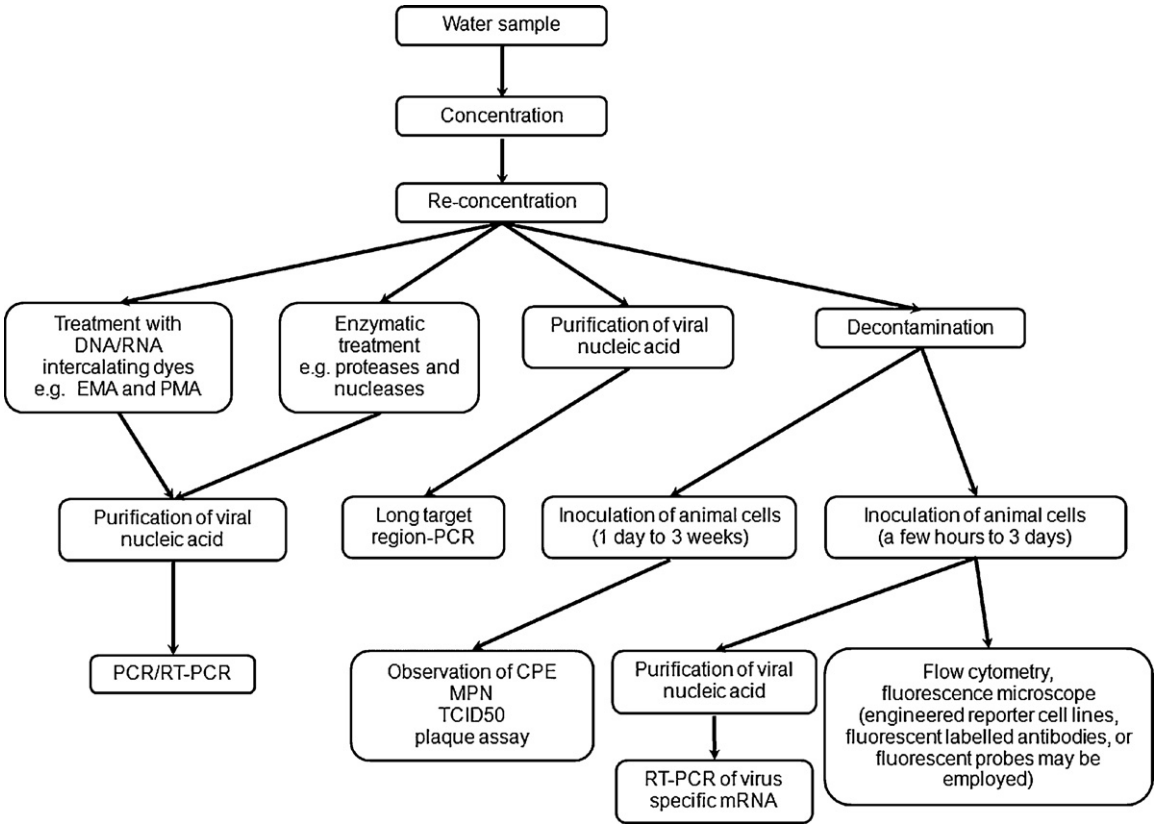

Fig. 1. Common approaches to estimate viral infectivity in environmental water samples.

the virus producing a CPE in 50% of the cultures (Reed and Muench, 1938).

The most frequently used assay for enumeration of infectious waterborne enteric viruses is the plaque assay (Ali et al., 2004; Calgua et al., 2011; Choi and Jiang, 2005; Li et al., 2010a; Pina et al., 1998; Tani et al., 1995). The method involves inoculation of the cell culture with virus suspension, followed by immobilisation with agar to keep growing plaques separated. One plaque is taken as being the progeny of one infectious virus particle. However, such counts should be interpreted with caution, especially when data are used in quantitative microbial risk assessment to estimate the public health impact (Teunis et al., 2005). It should be noted that many viruses do not form plaques and so they will not be detected under agar.

Basically, plaque assay, MPN and TCID50 are morphological assays that are not specific for a particular virus. Therefore, the viral pathogen should be confirmed with other approaches such as molecular or immunological assay. Additionally, the fact that more than one virus may co-infect cell culture should not be dismissed as the fast growing viruses may compete out the slow growing viruses. Other factors should be considered when using conventional cell culture based assay including the long incubation period required for some viruses to produce CPE, the inability of some viruses to proliferate in traditional cell cultures, and the cytotoxicity of compounds from the environmental sample which may either lead to false positive results due to CPE of the contaminating compounds or to reduction of the sensitivity of the cell culture assay due to impaired viability of the cells.

Long target region PCR to assess genome integrity

A sensitive PCR/qPCR usually targets a small portion of the viral genome. However, analyzing a long target region of the viral genome by RT-PCR was proposed to detect the integrity of viral genome that may correlate with virus infectivity (Allain et al., 2006; Li et al., 2002a; Simonet and Gantzer, 2006a,b; Wolf et al., 2009).

Since primer selection determines the portion of the viral genome that is amplified by (RT) PCR, in EV a poly (A) tail of 3′-untranslated regions can be targeted by a poly(T) primer to transcribe the entire viral genome (Simonet and Gantzer, 2006a). Hence, if the RNA is damaged, the reverse transcriptase detaches from the RNA and polymerization ceases.

It was hypothesized that the larger the genome region analyzed, the more likely the nucleic acid damage could be detected. The method was used to assess the inactivation of hepatitis A virus by chlorine (Li et al., 2002a). It has been found that different positions of the virus nucleic acid have different levels of resistance to chlorine and the inactivation effect of disinfectant on virus can be evaluated by PCR (Li et al., 2002a). Simonet and Gantzer (2006a) used this principle to investigate the influence of chlorine dioxide on extracted RNA and on the infectious poliovirus 1. RT-PCR was used to measure the degradation of genome fragments of different sizes and positions (76-base in the 3C region, 145-base fragment in the 5′-untranslated region (5′-UTR), 1869-base fragment in 3C region and 6989-base; nearly the entire viral genome). They found that general pattern of poliovirus 1 genome degradation decreased with decreasing fragment size (Simonet and Gantzer, 2006a). The study also revealed that relationship between degradation and size was not linear because degradation of 1869 bases of 3′-terminal fragment (oligo dT primer for RT and 3C region primers for PCR) was as rapidly as the 5′-UTR fragment with 145-bases (5′-UTR primers for RT and PCR). Furthermore, the infectious poliovirus was very rapidly inactivated by 5 mg/l chlorine dioxide for 5 min, however, the drop-off was more than $3\log_{10}$ for 6989-base fragment, which accounts for nearly the whole genome, and only $1.4\log_{10}$ for the 76-base fragment of 5′-UTR.

Long target region PCR was used to demonstrate the damage of poliovirus 1 and MS2 bacteriophage after UV-irradiation. Simonet and Gantzer (2006b) compared the capacity of the size of PCR target genome to detect changes in the RNA of poliovirus and coliphage MS-2 after UV irradiation. Although a linear correlation was observed between the rate of RNA degradation by UV and the fragment size, the size of the viral RNA was not the only factor affecting UV-induced RNA degradation. This was obvious since viral RNA was more rapidly degraded in poliovirus 1 than in the MS2 phage for a similar fragment size (Simonet and Gantzer, 2006b).

Similarly, the phenomenon was used to assess the genomic integrity of murine norovirus-1 and human norovirus after heat inactivation and UV exposure (Wolf et al., 2009). Although murine norovirus-1 was completely inactivated by heat treatment at 72 °C for 3 min, only limited effect on both short and long target region of RT-PCR was found under the same temperature. Long target qPCR also showed remarkable decrease with the increase of UV exposure time comparable to the decrease in the plaque assay, while the short target qPCR was not affected (Wolf et al., 2009).

These reports show that the detection of short genome fragments is insufficient to reveal the presence of the infectious virus. The position and/or the sequence of the detected fragment also have to be considered in addition to the fragment size to avoid an overestimation of the risk of viral infection using PCR-based approach. Nevertheless, the efficiency of PCR based assays can be decreased owing to the presence of PCR-inhibitory compounds in the environmental water samples. More importantly, inactivation of viruses is also possible without damaging the viral genome. Enveloped viruses loose infectivity by degradation of surface proteins, in the absence of detergents the integrity of the viral genome is not affected. Similarly cross-linking agents can fix the virus particle and thus destroy their infectivity without damage to the viral genome. This somehow limits the general applicability of long target region PCR as a surrogate marker for viral infectivity.

Integrated cell culture—PCR

Combination of cell culture with PCR has permitted detection of infectious viruses that grow slowly, or fail to produce CPE. The method relies on an initial biological amplification of the viral nucleic acids, followed by (RT-)PCR or real time (RT-)PCR amplifications.

Since integrated cell culture PCR (ICC-PCR) has the benefits of cell culture and PCR, it attempts to compensate for several disadvantages in the cell culture such as time consuming and limited detection sensitivity. ICC-PCR has been first proposed as an alternative method for detection of waterborne enteric viruses in environmental samples by Reynolds et al. (1996) and Murrin and Slade (1997). Using ICC-PCR, the presence of infectious EV was confirmed as early as 1 day post-inoculation in comparison to 3 days or more by traditional cell culture infectivity assay (Murrin and Slade, 1997; Reynolds et al., 1996). The use of ICC-PCR/ICC-qPCR has been described for the detection of a wide variety of human pathogenic viruses in aquatic environment such as enteroviruses, adenoviruses, rotaviruses, hepatitis A virus, astroviruses and reoviruses (Abad et al., 1997; Balkin and Margolin, 2010; Ballester et al., 2005; Blackmer et al., 2000; Chapron et al., 2000; Grimm et al., 2004; Hamza et al., 2011; Jiang et al., 2004; Lee et al., 2005; Li et al., 2010a; Reynolds, 2004; Rigotto et al., 2010; Shieh et al., 2008; Spinner and Di Giovanni, 2001).

ICC-PCR provides a more sensitive and rapid method for detection of infectious viruses following chlorine disinfection compared to conventional cell culture. The method was used to evaluate the effectiveness of poliovirus inactivation by chlorine, so it could determine the potency of the chlorine disinfection, minimizing the chance of false-negative results due to single passage cell culture alone (Blackmer et al., 2000). Using this approach, the resistance of rotavirus to UV disinfection was demonstrated (Li et al., 2009). Li and his group compared ICC-qRT-PCR to conventional cell culture assay and qRT-PCR assay. The data obtained by ICC- qPCR provided more detailed assessment of virus resistance to UV disinfection at much wider dosage range compared to conventional cell culture assay. As a result, the authors pointed out that conventional cell culture assay may underestimate the UV dosage required to achieve certain level of inactivation (Li et al., 2009). At the same study, rotavirus genomes were found to be quite persistent under UV disinfection so the results of qRT-PCR did not correlate with virus infectivity. Similarly, ICC-qPCR method was proposed as a practical alternative for the quantification of adenoviruses in disinfection studies (Gerrity et al., 2008). Whereas, the detection reliability of indigenous viruses in environmental water samples by ICC-PCR can be restricted by the primer sets that are used (Lee and Jeong, 2004).

The detection of (−)RNA initiated by a positive strand (+)RNA virus as a replicative-intermediate and marker of infectivity (Belsham and Normann, 2008) was also suggested to differentiate between infectious and non-infectious viruses. The detection of the (−)RNA was used in clinical samples for the detection of infectious viruses such as hepatitis C (Carriere et al., 2007) and enteroviruses (Li et al., 2002b). The method was recently used to detect infectious hepatitis A virus in environmental water samples at sensitivity of one infectious unit per cell culture flask within 4 days of incubation (Jiang et al., 2004). Although ICC-PCR reduces the time needed for detection of infectious viruses and overcome the sensitivity of direct PCR reaction to inhibitors of water samples, this technique may be costly and carryover detection of the DNA of inactivated viruses inoculated onto cultured cells is possible.

Detection of virus specific mRNA

The infectivity of DNA viruses that do not replicate well in cell culture may be determined by the detection of a number of early and late genes in infected cells. To avoid the contamination by viral DNA genome during the RT-PCR, primers targeting the spliced forms of the gene (El Hassan et al., 2006) or DNase treatment prior to cDNA synthesis can be used.

Enteric adenoviruses are difficult to culture and do not produce clear and consistent CPE. Sensitive detection of infectious human adenoviruses by RT-PCR assay of mRNA followed by nested PCR was developed (Ko et al., 2003). HAdV 2 mRNA was detected as early as 6 h post-infection and HAdV 41 was detected 1 day after infection of A549 cells. In this approach the incubation time of samples on the cells depends on virus type, cell type, and target gene. Moreover, the authors modified the protocol by use of TaqMan real-time RT-PCR method in conjunction with cell culture infectivity to rapidly detect mRNA produced by infectious HAdV in water samples (Ko et al., 2005). Detection of HAdV mRNA in infected cells demonstrated that HAdV 41 is more resistant to UV radiation than in a previous study using a conventional cell culture infectivity assay.

Detection of viral specific mRNA of DNA viruses can be used therefore to determine the efficiency to inactivate some DNA viruses during water treatment process and the infectivity of indigenous viruses in environmental samples. Whereas, carryover detection of the DNA of inactivated viruses inoculated onto cultured cells is possible.

Immunomagnetic separation and virus infectivity

Immunomagnetic separation (IMS) has been commonly used to recover enteric viruses such as hepatitis A virus, norovirus, rotavirus and enterovirus from environmental water samples followed by PCR (Casas and Sunen, 2002; Gilpatrick et al., 2000;

Grinde et al., 1995; Jothikumar et al., 1998; Monceyron and Grinde, 1994; Myrmel et al., 2000). More recently real time PCR and real time NASBA was also used coupled to IMS to detect hepatitis A virus (Abd El Galil et al., 2004, 2005), rotavirus (Yang et al., 2011), adenovirus and human polyomavirus (Haramoto et al., 2010) in environmental water samples.

In this method, antibody coated paramagnetic beads will bind to antigens present on the surface of the target pathogen and facilitate the concentration of this bead-attached pathogen by a magnet (Fig. 2). The method was proposed to reduce the co-purification of PCR inhibitors from the environmental samples as well as for selective detection of viral particles, rather than naked viral genomes. The ability to discriminate between infectious and non-infectious viruses depends on antigenic properties of the viral capsid. The method relies on the damage in the viral capsid may change the antigenic properties of the virus, and specific viral antigen–antibody complexes may not form, leading to negative IMS-PCR results.

IMS real time PCR was compared to plaque assay to detect infectious EV in seeded surface water. Hwang et al. (2007) reported that IMS provided the same detection sensitivity as the conventional plaque assay. A recent report by Yang et al. (2011) demonstrated that IMS combined with quantitative real-time reverse transcription (RT)-PCR is an effective and sensitive detection method of rotaviruses in water environments. The approach was successfully applied to purify and detect rotavirus particles seeded in concentrated wastewater samples. Also the method was compatible with commonly used virus eluants, including beef extract, beef extract with 0.05 M glycine and urea arginine phosphate buffer. Importantly, different reports showed that the efficiency of IMS is affected by sample matrix (Monceyron and Grinde, 1994), kind of water (Grinde et al., 1995) and pH value (Kuhn et al., 2002).

One of the limitations of IMS for the enrichment of human enteric viruses from water samples is that the antibody may be not able to target all possible strains of the virus under study or all enteric viruses, so it requires specific assay for each virus. Additionally, the relative sensitivity of antigen-binding affinity to several factors, including amount of colloidal particles and pH value of water sample should not be neglected. Besides, the cost of magnetic beads separation can be higher than other methods, depending on the reagents coupled to the beads.

Enzymatic treatment of water samples before PCR

Although the virion concentration step from water is mainly based on physical properties of viral particles, it is difficult to draw a clear correlation between the genome copy number obtained by PCR and the infectivity assay. The loss of some of the viral protein may alter the infectivity but the nucleic acid itself could be still protected and detectable by molecular tools. Nuanualsuwan and Cliver (2002) proposed the pre-treatment of virus containing water sample with proteinase K and RNase for the detection of the intact viral particles during three modes of virus inactivation; heat, ultraviolet and sodium hypochlorid. As a mean of preventing a positive RT-PCR test in the absence of infectious virus, Nuanualsuwan and Cliver showed that the intact viral capsids were less susceptible to protease degradation than the damaged capsid. Consequently, protease pre-treatment degrades the capsid damaged by disinfection, allowing the nuclease pre-treatment to degrade the unprotected nucleic (Nuanualsuwan and Cliver, 2002).

The enzymatic treatment prior to extraction of viral nucleic acid was useful for viruses that were inactivated by treatment at 72 °C, with chlorine, and with UV but not for viruses that were inactivated by long exposure at 37 °C in which viral capsids still protect the viral nucleic acid (Nuanualsuwan and Cliver, 2003).

Baert et al. (2008) used the pre-enzymatic treatment approach to examine the infectivity of murine norovirus following heat exposure to 80 °C. They reported that heat inactivation had a much stronger detrimental effect on virus infectivity than on the integrity of the viral genome. Reductions of more than 6 $\log_{10}$ units as determined by a plaque assay was found but at the same time, more than 9 $\log_{10}$ genomic copies were detected by real-time RT-PCR (Baert et al., 2008). Nevertheless, the reductions in viral RNA copy numbers may only be related to reduction of infectivity under extreme conditions of inactivation (Duizer et al., 2004).

The pre-enzymatic digestion-PCR was used to demonstrate that viral nucleic acids are not stable in sewage water for long periods (Bofill-Mas et al., 2006). These workers demonstrated that the time required for a reduction of 99% from the initial viral concentration (T_{99}) was 132.3 days for HAdV and 127.3 days for JCPyV at 20 °C. Whereas, treatment of viral concentrate with DNase prior to the nucleic acid extraction, showed that the T_{99} for HAdV and JCPyV was 126.1 and 121.4 days, respectively (Bofill-Mas et al., 2006). Similarly, Hamza et al. (2009b) observed no decrease in the human bocavirus genome copy numbers with and without a DNase treatment before nucleic acid extraction, which suggested that viral DNA was protected in an intact capsid.

Indeed, if a strong association can be demonstrated between loss of infectivity and susceptibility to the pre-enzymatic digestion, this approach can be used to overcome the drawbacks of the conventional cell culture based virus infectivity assay. However, the presence of PCR inhibitors in environmental samples can reduce the sensitivity of this protocol.

Dye treatment of water samples before PCR

Different dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) have been used for selective detection of viable bacteria (Bae and Wuertz, 2009a,b; Chang et al., 2010; Chen and Chang, 2010; Kobayashi et al., 2010; Lin et al., 2011; Nocker et al., 2006, 2010; Nogva et al., 2003; Pan and Breidt, 2007; Rudi et al., 2005; Singh et al., 2011; Wang et al., 2009), protozoa (Bertrand et al., 2009; Brescia et al., 2009; Sauch et al., 1991) and fungi (Andorrà et al., 2010; Vesper et al., 2008). EMA and PMA are closely related DNA intercalating dyes with a photo-inducible azide group that covalently cross-links to DNA after photo activation (Nocker et al., 2006). The dye can enter only bacterial cells with compromised cell walls and cell membranes (Rudi et al., 2005). Such modified DNA structure leads to a strong signal reduction in subsequent real-time PCR, as a result of PCR inhibition. However, few studies have found that EMA-PCR is a poor indicator of cell viability, since the use of EMA prior to DNA extraction may lead to a significant loss of the genomic DNA of some viable bacteria (Flekna et al., 2007). While, propidium monoazide (PMA) is a more selective agent, penetrating only dead bacterial cells but not viable cells with intact membranes (Nocker et al., 2006). In bacteria this approach could not monitor the loss of viability as a result of UV exposure, owing to UV light causes DNA damage without directly affecting membrane (Nocker et al., 2007). It is also possible to observe the same effect with UV-treated viruses.

Recently, few studies have examined the possibility of dye interacting DNA/RNA as a tool to distinguish between infectious and inactivated viruses (Graiver et al., 2010; Kim et al., 2011; Parshionikar et al., 2010). Graiver et al. (2010) measured the survival rate of avian influenza viruses in water using cell culture titration and real time RT-PCR with and without EMA pre-treatment. Lower titres of viruses were obtained by cell culture than both RT-PCR procedures. Moreover, no significant differences were found between RT-PCR results with and without the EMA pre-treatment step, thus EMA may not bind effectively to RNA in

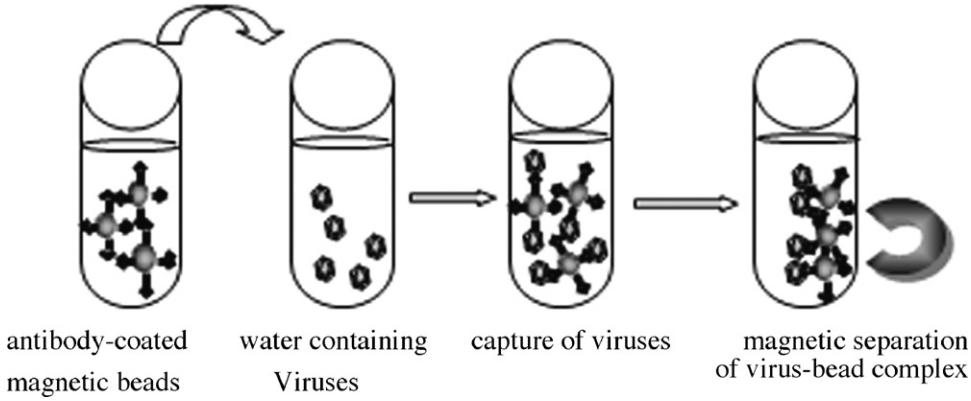

Fig. 2. Schematic representation of the use of antibody coated paramagnetic beads for separation of viral particles from water.

inactive virions. It was also found that EMA may not bind effectively to extracted RNA from avian influenza viruses (Graiver et al., 2010). Although EMA did not distinguish between infectious and non-infectious avian influenza virus particles, the results of EMA-RT-PCR were comparable to the plaque assay of poliovirus 1 after thermal inactivation at 45 °C, 55 °C, 65 °C and 75 °C (Kim et al., 2011). However, in these studies different conditions of the experiment such as EMA concentration, incubation temperature and different viruses were used.

Likewise, pre-treatment of enteric virus suspension (coxsackievirus B5, echovirus 7, poliovirus, and norovirus) with PMA prior to RT-PCR may allow for the discrimination between infectious and non-infectious virus particles, particularly when virus particles are inactivated by heating at 72 °C or 37 °C or by hypochlorite. Nevertheless, PMA-RT-PCR was unable to prevent detection of norovirus and enteroviruses that were rendered non-infectious by treatment at 19 °C (Parshionikar et al., 2010). PMA-qPCR was not sufficiently effective to distinguish between infectious and non-infectious viruses even after heat inactivation (85 °C) and protease treatment (Fittipaldi et al., 2010) as shown in Fig. 3. Although the data from the PMA-qPCR method were in better agreement with the cell culture data than the qPCR, the PMA-qPCR method resulted in a positive signal amplification (Fittipaldi et al., 2010), which can yield false positives. In contrast, under higher inactivation temperature (110 °C), a clear discrimination of infectious and non-infectious viruses was observed with PMA-qPCR.

The less concordance between studies demonstrating the suitability of EMA-(RT-)PCR or PMA-(RT-)PCR for the discrimination of infectious and non-infectious viruses suggests that this approach has to be carefully adapted to each specific target organism. Optimization of EMA or PMA concentration, time exposed to the light source, distance from the light source, and light source may lead to a more effective PMA/EMA pre-treatment. Likewise the pre-enzymatic treatment-PCR approach, PMA-(RT-)PCR and EMA-(RT-)PCR depend mainly on capsid integrity as the criterion for the infectivity, and this could be one of the drawbacks of this technique since virus inactivation may take place by means other than membrane or particle disruption. This was obvious when PMA-RT-PCR failed to discriminate between viruses that lost infectivity at 19 °C because their capsids did not allow penetration of PMA. In addition, the residual PMA/EMA may lead to PCR inhibition which needs extra purification steps before/after nucleic acids extraction.

Flow cytometry and virus infectivity

Flow cytometry (FC) is an excellent method for rapid, high-throughput detection and quantitation of infected cells from large cell populations. Like most of recently developed methods for virus detection, it may have a wide use to detect different viruses in clinical applications and drug discovery. FC-based methods may employ fluorescent dyes, fluorochrome labelled antibodies, or fluorescent proteins to analyze virus-infected cells.

The use of FC with environmental samples is limited. FC was proposed to monitor the presence of rotavirus in environmental samples without the need for prolonged periods of adaptation to growth in tissue (Abad et al., 1998; Barardi et al., 1998). The development of this method relied on the use of the CaCo-2 cell line or MA104 for the propagation of rotavirus and the detection by FC using labelled antibodies.

Alternative approach was proposed to monitor the replication of EV in cell culture by the use of engineered reporter cell lines, which can be designed to exhibit specific characteristics upon infection. Cantera et al. (2010) used a cell based reporter system of engineered BGMK cells which expressing the cyan fluorescent protein (CFP)–yellow fluorescent protein (YFP) substrate linked by a cleavage recognition site for poliovirus 2A protease. A fluorescence protein pair undergoing fluorescence resonance energy transfer (FRET), in which fluorescence energy is transferred from an excited fluorophore (reporter) to a light-absorbing molecule (acceptor) when located in close proximity (Fig. 4). The reporter system of engineered BGMK cells was further combined with FC for the quantification of the infectious poliovirus. The number of infected cells was determined by counting the number of cells with an increased CFP-to-YFP ratio. As early as 5 h postinfection, a significant number of infected cells was detected by flow cytometry, and cells infected with only 1 PFU were detected after 12 h postinfection (Cantera et al., 2010). To demonstrate the potential of the FC-based assay for environmental applications, spiked water runoff sample with known titres of poliovirus 1 was used. They found that FC-based assay could provide a level of sensitivity similar to that of the plaque assay for detecting and quantifying infectious virus particles (Cantera et al., 2010).

Li et al. (2010a,b) developed fluorescence-activated cell-sorting (FACS) assay to detect infectious HAdV in animal cell culture. The assay based on monitoring the expression of viral protein during replication in cells with specific fluorescently labelled antibodies. As little as 1 PFU HAdV was detected by FACS within 3 days post-infection. The assay was applied to detect HAdV in sewage samples. Adenovirus was detected in 4/6 of primary effluent and 4/12 of the secondary effluent after a 3-day incubation period (Li et al., 2010b).

Although, it significantly reduces the time used for detection of infectious viruses in environmental samples and assess viral stability and inactivation as a result of water treatment and disinfection, the method needs higher cost and highly trained operators for routine application. Moreover, it does not overcome the obstacles to

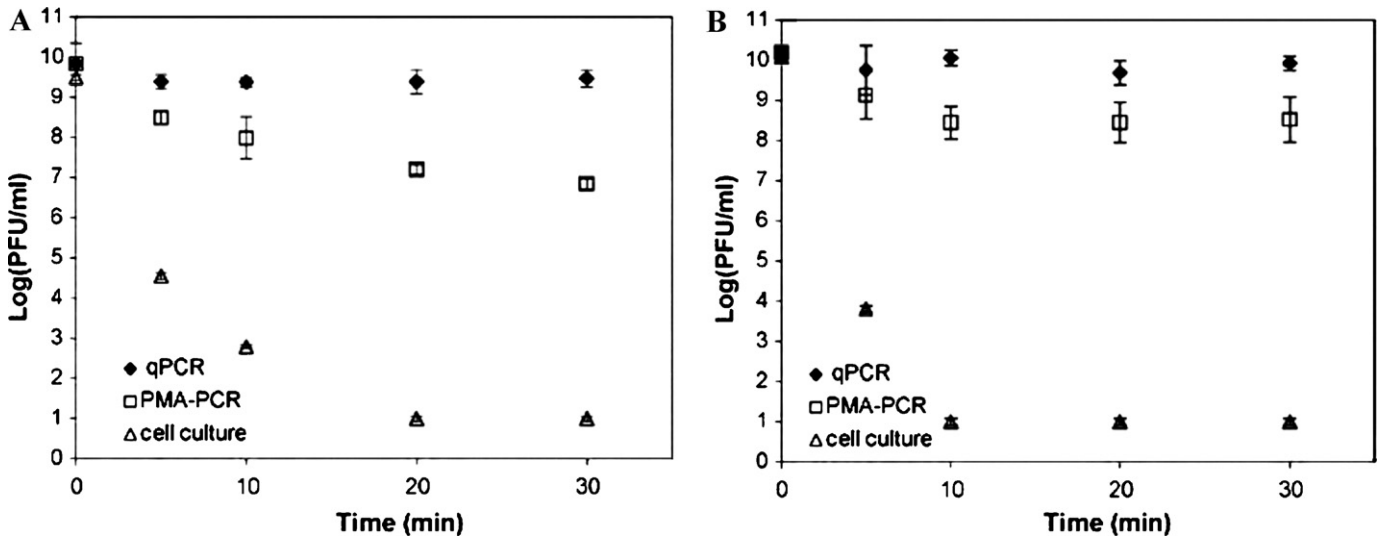

Fig. 3. Effect of (A) heat treatment (85 °C) and (B) protease treatment on bacteriophage T4 quantitation over time as assessed by plaque assay, qPCR, and PMA-PCR. As shown, PMA-qPCR is not sufficiently effective to discriminate between infectious and non-infectious viruses. Error bars show standard deviations from three independent assays. Figure adapted from Fittipaldi et al. (2010) with permission.

investigate the infectivity of viruses that do not infect cell cultures such as human noroviruses.

Fluorescence microscopy and virus infectivity

Immunofluorescence assay (IFA) has been used with several types of viruses mainly in the clinical field (Rigonan et al., 1998; Terletskaia-Ladwig et al., 2008). This method uses virus-specific antibody labelled with a fluorescent dye to visualize cell-associated viral antigens (Jeffery and Aarons, 2009). The use of the IFAs has been described previously for detecting infectious rotaviruses in sewage (Ridinger et al., 1982; Smith and Gerba, 1982). Smith and Gerba (1982) have shown that as few as 10 infectious units per liter can be reliably detected by IFA within less than 24 h incubation. Ridinger et al. (1982) found that IFA is more sensitive and more rapid than the plaque assay for the detection of reoviruses. Reoviruses in excess of 10^4 per liter of raw sewage were detected by IFA. During this study, the authors found that the efficiency of the assay depends mainly on the sensitivity of the cell line to the reoviruses. The comparison of IFA to the classical plaque assay showed that reoviruses detection could be possible within 10 h post-infection by IFA, whereas the formation of plaques requires 4–7 days.

To eliminate the need for expensive antibodies, the laborious steps in immunolabeling procedures, and the difficulties in internalization the antibody inside the cells, an engineered fluorescent reporter system was described in conjunction with the fluorescent microscope to monitor the change in FRET as a result of virus activity (Hwang et al., 2006). More recently, IFA was described by Calgua et al. (2011) to detect the infectious form of two most commonly detected viruses in water, HAdV and HPyV (Calgua et al., 2011). IFA was compared to the most commonly used methods for virus detection such as plaque assay, tissue culture infectious dose-50 (TCID50) and quantitative real time PCR. The IFA was found to be specific for the detection of infectious viruses. The method showed higher sensitivity for the detection of infectious viral particles than other cell culture techniques evaluated. Moreover, negative results were obtained by IFA when UV or heat-inactivated viruses were analyzed. Since the assay is based on the sensitivity of the cell type to virus infection, the optimal reading days for the IFAs were day 4 for HAdV 2 and day 8 for HAdV 41 and JCPyV. The developed IFA detected 1 $\log_{10}$ more infectious viruses for HAdV 2 than the plaque assay and TCID50. Also the values in focus-forming unit per

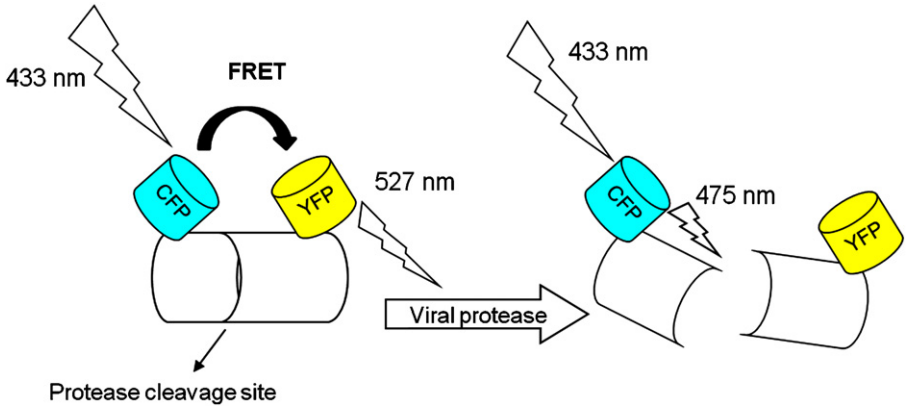

Fig. 4. Schematic representation of FRET for monitoring viral proteolytic activity in the infected cells. The cyan fluorescent protein (CFP)–yellow fluorescent protein (YFP) substrate is linked by a cleavage recognition site for poliovirus 2A protease. A fluorescence protein pair undergoing FRET, in which fluorescence energy is transferred from an excited fluorophore (reporter) to a light-absorbing molecule (acceptor) when, located in close proximity. A disruption of FRET, as shown by a change in CFP and YFP emission signals, indicates active viral replication within the infected cells.

Table 3
Pros and cons of some approaches to assess virus infectivity in water.

Approach	Pros	Cons	References
Long target region-PCR	Easy; may reveal the presence of damaged viral genome	Each targeted fragment may have different sensitivity to the disinfectant; sole use of genome integrity as the criterion for the infectivity; PCR of LTR may have low sensitivity.	Allain et al. (2006); Li et al. (2002a); Simonet and Gantzer (2006a,b); Wolf et al. (2009)
Integrated cell culture-PCR	More sensitive and faster than cell culture alone; less susceptible to PCR inhibitors	Costly; carryover detection of nucleic acid of inactivated viruses; non-appropriate for non-culturable viruses	Reynolds et al. (1996)
Detection of virus specific mRNA and negative strand RNA of positive RNA viruses	Faster than conventional cell culture; less susceptible to PCR inhibitors	Costly; carryover detection of nucleic acid of inactivated viruses; non-appropriate for non-culturable viruses, specific to certain type of viruses	Ko et al. (2003, 2005)
Nuclease and proteinase treatment before PCR	Rapid; may confirm damaged viral capsid; no need for cell culture	Unable to assess the thermal inactivation that may occur at 37 °C; use capsid integrity as the criterion for the infectivity	Nuanualsuwan and Cliver (2002, 2003)
Dye treatment of water samples before PCR	Rapid; may confirm damaged viral capsid; no need for cell culture	Use capsid integrity as the criterion for the infectivity; possible PCR inhibition by the residue of the dye; was unable to detect thermal inactivation of enterovirus at 19 °C	Graiver et al. (2010); Kim et al. (2011); Parshionikar et al. (2010)
The use of flow cytometry	Rapid; sensitive; high-throughput detection; automated	Expensive, non-appropriate for non-culturable viruses	Abad et al. (1998); Barardi et al. (1998)
The use of fluorescence microscopy	Rapid, sensitive; may study the real-time monitoring of viral replication	Expensive antibodies may be needed, false positive results may be obtained owing to short life time of the MB probe.	Calgua et al. (2011); Hwang et al. (2006); Ridinger et al. (1982); Smith and Gerba (1982); Yeh et al. (2008a,b)
Immunomagnetic separation of viral particles	Selective detection of virus particles; the effect of PCR inhibitor is minimal	Depends on the antigenic properties of the virus, antibody may be not able to target all possible strains of virus; may be costly	Casas and Sunen (2002); Gilpatrick et al. (2000); Grinde et al. (1995); Haramoto et al. (2010); Jothikumar et al. (1998); Monceyron and Grinde (1994); Myrmel et al. (2000)
Measurement of the capsid carbonyl content	Sensitive, may demonstrate the decrease in infectivity of non-culturable viruses	Insufficient to reveal the presence of the infectious viruses; costly	Sano et al. (2010)

ml obtained by the IFA were approximately 2 $\log_{10}$ less than the values in genome copies per ml obtained by qPCR (Calgua et al., 2011).

A different approach based on the detection of the newly synthesized viral nucleic acid by molecular beacon combined cell culture assay was described (Yeh et al., 2008a). Molecular beacons (MBs) are single stranded hairpin shaped oligonucleotide probes, labelled at the 5′ and 3′ ends with reporter and quencher dye, respectively. In the presence of the target sequence, they unfold, bind and fluoresce. Yeh et al. (2008a) used hepatitis A virus as a surrogate for enteric viruses. The detection of infectious HAV with a detection limit of 1 PFU within 6 h post-infection was achieved so the reported fluorescence assay combines a 32-fold reduction in detection time with a quantitative capability similar to that for the 8-day conventional plaque assay for HAV at the same cells. Furthermore, the utility of the assay for the detection of HAV in surface water samples was demonstrated in parallel to plaque assay, showing that the infectious dosages obtained from the two assays were remarkably similar with a maximum of 25% difference (Yeh et al., 2008a).

However, when long term real time monitoring of viral replication is needed, the use of MBs can show false positive results. This could be due to the relatively short half-life (~30 min) of MBs due to endogenous nuclease degradation. Thus, a modified protocol was proposed to use a nuclease-resistant MB containing 2′-O-methyl RNA bases with phosphorothioate internucleotide linkages (Yeh et al., 2008b). To enhance the intracellular delivery, a cell-penetrating TAT peptide was conjugated to the MB by using a thiol–maleimide linkage. When the conjugate was introduced into BGMK cell monolayers infected with coxsackievirus B6, a discernible fluorescence was observed within 30 min post-infection, and as few as 1 infectious viral particle could be detected within 2 h (Yeh et al., 2008b). This method is particularly attractive when applied to viruses that grow poorly in cell culture and those that do not produce detectable CPE in infected cells. While, it will probably not solve the problem of monitoring virus infectivity for viruses that do not infect cell cultures such as human noroviruses.

Detection of oxidative products on viral capsid protein

A new approach to measure the integrity of viral capsid protein was proposed particularly for non-culturable viruses such as noroviruses (Sano et al., 2010; Wei et al., 2007). The protocol depends on the direct detection of the oxidative damage in the viral capsid protein as a result of exposure to disinfectants or environmental stresses.

Residues of some amino acids, including lysine, arginine, proline, and threonine can be oxidized by many chemicals, and may also yield carbonyl derivatives which attendant loss of biological function. The presence of carbonyl groups in proteins has therefore been used as a marker of protein oxidation (Levine et al., 1994).

Cumulative oxidative damages on viral particles were detected by biotinylation of oxidized proteins with biotin hydrazide followed by affinity enrichment using monomeric avidin affinity chromatography columns (Mirzaei and Regnier, 2005).

Theoretically, a carbonyl group does not exist in intact capsid proteins, therefore Sano et al. (2010) demonstrated that the detection of the carbonylated viral particles could be a promising tool for the evaluation of the decrease in the infectivity of non-cultivatable viruses. A positive correlation between the degree of oxidative damage on human astrovirus by free chlorine and the infectivity

assay was found. The accumulation of carbonyl groups on norovirus particles was also quantitatively evaluated. A 5-fold increase of human norovirus recovered as oxidatively damaged particles after treatment with 1 ppm free chlorine for 15 min over viral particles that were treated with 0.5 ppm free chlorine for 15 min. Therefore, the method might be a powerful tool to demonstrate the decrease in infectivity of non-culturable such as noroviruses. Knowing the mode of action of most commonly used disinfectants, similar approaches can be used to identify reactions/changes that can occur in virus capsid proteins upon treatment (Rule Wigginton et al., 2010).

The purification by affinity chromatography is attractive owing to the selectivity and the binding sites of biological molecules can be easily investigated, so high virus binding capacity can be obtained. Though sensitive, if more details about the inactivation sites are needed, affinity chromatography is unable to identify and distinguish between different oxidization sites which may contribute to inactivation at varying degrees. Whereas, matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) mass spectrometries (MS) can be used for this purpose (Rule Wigginton et al., 2010).

Although, the measurement of carbonyl content of viral capsid proteins can be used to demonstrate oxidative effect on the capsid protein, the absence of oxidative damage may not reveal that viruses still infectious because other mechanisms may lead to virus inactivation in aquatic environment. Moreover, the high cost of its application may hinder the wide use in environmental virology particularly in routine work.

Conclusions

Presently, nucleic acid amplification techniques such as PCR and real time PCR are the most commonly applied to monitor viral contamination in aquatic environment. Molecular tools are rapid, highly sensitive and specific, and they may provide valuable information for epidemiological studies via genotyping. However, a lack of correlation between the detected genome copy numbers of target viral pathogens and their infectivity calls for the development of alternative approaches to assess the infectivity of the pathogenic viruses in environmental samples. The only reliable method to detect infectious viruses is based on mammalian cell culture, which monitors the production of visible CPE, but lacks general specificity. Moreover, conventional cell culture is not practical to assess the infectivity of some health significant viruses that are slow or non-cultivable. Therefore, several alternatives have been proposed to overcome the drawbacks of the conventional cell culture infectivity assay, the advantages and disadvantages of these approaches are summarized in Table 3.

Combination between the molecular tools and cell culture allowed for sensitive and fast detection of virus infectivity in environmental water samples, particularly for slow growing viruses. Although it seems possible to use this method to determine the infectivity of human enteric viruses in environmental samples, the absence of a universal cell line that can be used to propagate a wide range of human enteric viruses hinders its wide application. The same obstacle may also face the conjunction of cell culture with flow cytometry and fluorescent microscopy based protocols, so new cell lines need to be investigated.

The use of capsid integrity as a marker of viral infectivity was also suggested. Different approaches such as enzymatic digestion, dye treatment prior nucleic acid extraction and direct detection of the oxidative lesions showed promising results to discriminate between infectious and non-infectious viral particles. However, virus inactivation may occur by mechanisms other than the disruption of the capsid protein and the mode of inactivation may be temperature dependent. Thus to differentiate between potentially infectious and non-infectious viruses more evaluations are still needed before interpretation of PCR results combined with dye or enzymatic pretreatment of virus suspension.

The optimal method to assess virus infectivity should be technically easy to accomplish in a short time, applicable to a wide range of viruses and not be costly. It is obvious from the several approaches stated in this review that no single method can fulfil these criteria. Each method has drawbacks which prevent it from being optimal for routine lab work. Therefore, the development of new approaches is still needed. Next generation of detection of virus infectivity in environmental water samples may depend on the interesting, fast growing field of nanobiotechnology that has many applications in clinical diagnosis. The method has to provide high sensitivity, particularly few viral particles are expected to be found in contaminated environmental samples.

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