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Critical issues in application of molecular methods to environmental virology

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ABSTRACT

Waterborne diseases have significant public health and socioeconomic implications worldwide. Many viral pathogens are commonly associated with water-related diseases, namely enteric viruses. Also, novel recently discovered human-associated viruses have been shown to be a causative agent of gastroenteritis or other clinical symptoms. A wide range of analytical methods is available for virus detection in environmental water samples. Viral isolation is historically carried out via propagation on permissive cell lines; however, some enteric viruses are difficult or not able to propagate on existing cell lines. Real-time polymerase chain reaction (qPCR) screening of viral nucleic acid is routinely used to investigate virus contamination in water due to the high sensitivity and specificity. Additionally, the introduction of metagenomic approaches into environmental virology has facilitated the discovery of viruses that cannot be grown in cell culture. This review (i) highlights the applications of molecular techniques in environmental virology such as PCR and its modifications to overcome the critical issues associated with the inability to discriminate between infectious viruses and nonviable viruses, (ii) outlines the strengths and weaknesses of Nucleic Acid Sequence Based Amplification (NASBA) and microarray, (iii) discusses the role of digital PCR as an emerging water quality monitoring assay and its advantages over qPCR, (iv) addresses the viral metagenomics in terms of detecting emerging viral pathogens and diversity in aquatic environment. Indeed, there are many challenges for selecting methods to detect classic and emerging viruses in environmental samples. While the existing techniques have revealed the importance and diversity of viruses in the water environment, further developments are necessary to enable more rapid and accurate methodologies for viral water quality monitoring and regulation.

1. Introduction

Promoting good water quality is a major policy priority world-wide as water safety and public health are compromised by water-borne diseases. Fecally-contaminated water is a vehicle to transport human microbial pathogens and results in hundreds of millions of illnesses globally annually (Ashbolt, 2015; Shuval, 2003). Current microbial water quality monitoring approaches focus primarily on fecal indicator bacteria. Although bacteria are a major cause of diarrhea transmitted through contaminated water, viruses are also associated with waterborne transmission (Grabow, 2007) and account for the majority of predicted waterborne disease under specific exposure scenarios (Ahmed et al., 2018; Boehm et al., 2015).

The most significant entry routes for human enteric viruses into the aquatic environment are sanitary/combined sewer overflow and untreated discharges from wastewater treatment plants (WWTP), in addition to runoff from urban and suburban areas, and seepage or leachate from old or poorly maintained septic systems that contribute in

Abbreviations: A549 cells, human lung carcinoma cells; AdV, adenovirus; AMV, avian myeloblastosis virus reverse transcriptase; AstV, astrovirus; BGM, buffalo green monkey kidney cells; BKPyV, BK polyomavirus; cDNA, complementary DNA; ddPCR, droplet digital PCR; dPCR, digital PCR; dNTPs, deoxynucleotide triphosphate; EMA, ethidium monoazide; EV, enterovirus; FCV, feline calicivirus; FRET, fluorescence resonance energy transfer; GPP, gastrointestinal pathogen panel; HAV, hepatitis A; HEV, hepatitis E; HPyV, human polyomavirus; ICC-PCR, integrated cell culture PCR IMS immuno magnetic separation; JCPyV, JC polyomavirus; MA104 cells, simian kidney epithelial cells; MNV, murine norovirus; MPN, most probable number; NASBA, nucleic acid sequence based amplification; NGS, next generation sequencing; PFU, plaque forming unite; PMA, propidium monoazide; qPCR, realtime-PCR; PV, poliovirus; RdRp, RNA-dependent RNA polymerase; RT, reverse transcriptase; RoV, rotavirus; TCID50, 50% tissue culture infectious dose; TTV, torque teno virus; UV, ultraviolet; WHO, World Health Organization; WTA, whole transcriptome amplification; WWTP, wastewater treatment plant

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viral dissemination. Globally, less than 20% of wastewater is treated before being released back into the environment (WWAP, 2015). In addition, wastewater treatment does not typically remove all pathogenic viruses; as a consequence, enteric viruses can contaminate waters receiving WWTP effluent (Gantzer et al., 1998; Hamza et al., 2011a; Myrmel et al., 2006; Pinto et al., 2007). Enteric viruses target the cells of the gastrointestinal tract and are excreted in the fecal material of the infected persons. The World Health Organization (WHO) has classified waterborne viruses as having "moderate to high significance on the human health" (WHO, 2017). The list includes adenoviruses (AdV), enteroviruses (EV) such as coxsackieviruses and polioviruses (PV), hepatitis A (HAV) and E viruses (HEV), rotaviruses (RoV), astroviruses (AstV), and noroviruses (NoV). Enteric viruses are released in large numbers, up to 10^{11} per gram in the stools of infected persons, and have high persistence rates in water facilitating their waterborne transmission (Fong and Lipp, 2005).

The majority of enteric viruses are specific to humans; however, some viral species or genera may infect animals as well. The only known zoonotic human enteric virus is HEV. HEV has strains that infect both humans and animals including swine, goats, cattle, and rodents. Accordingly, some of the later animals may serve as HEV reservoir of some strains that could infect humans (Grabow, 2002). In addition, viruses that may not be considered enteric viruses, such as the respiratory viruses, are also excreted in either feces or urine. For example, respiratory adenoviruses have been shown to be abundant in sewage and transmitted by recreational waters (Bibby and Peccia, 2013b; Mena and Gerba, 2009); therefore, there is the potential for viruses that may not primarily replicate in the intestinal tract to be transmitted via water (Bofill-Mas et al., 2006; McQuaig et al., 2009).

Virus levels in water are typically too low for detection by direct analysis; thus, the detection of viruses in the aquatic environment often involves viral enrichment or concentration followed by assay on permissive cell lines or using a molecular tool. The concentration step recovers viruses in volumes ranging from less than 1 to greater than 1000 liters based on the water type. A reliable concentration method should be technically feasible, have a high recovery rate, provide a small volume of the concentrate, be cost effective, and be suitable for the diverse array of viruses that may be found in the water environment. Various concentration methods are available to recover viruses from environmental samples and have been recently reviewed elsewhere (Cashdollar and Wymer, 2013; Haramoto et al., 2018; Ikner et al., 2012). Viruses can be enriched from environmental samples via different methods such as virus adsorption elution (VIRADEL), entrapment, and coagulation/flocculation.

The VIRADEL method, which uses charged membranes or filters, is most commonly used for virus concentration from environmental water samples. VIRADEL depends primarily on the ionic charge of the filter and viral particles. In general terms, the virus will adsorb onto the collection filter under specific conditions of pH and ionic strength, followed by viral elution using appropriate elution buffer. However, the volume of the elution buffer is often too large to be analyzed directly, particularly when molecular studies are needed. The type of matrix used, the elution buffer and type of water may have influence on the final volume of the elution buffer. The most commonly used buffers for viral elution from the matrix are alkaline solutions of beef extract, and glycine/NaOH. One major advantage of the VIRADEL procedure is that filters can be applied for simultaneous recovery of viruses, bacteria and parasites from water samples (Ali et al., 2004; Haramoto et al., 2011; Polaczyk et al., 2007). The disadvantage of using the VIRADEL method is that filters can be clogged by water samples with high turbidity. Hence, sample prefiltration or clarification steps could be used, but this must be validated to avoid viral loss prior to the adsorption step.

Negatively charged cellulose nitrate membranes are widely used due to their availability in various diameters, pore sizes, configurations and compositions. The virus binds to the filter by electrostatic forces. To enhance electrostatic binding of virus to the filter, the pH of water samples is adjusted to 3.5 and multivalent cations (eg. $MgCl_2$, $AlCl_3$) may also be added (Wallis and Melnick, 1967). Currently this method is widely used for virus recovery from surface waters as well as sewage treatment effluent and diluted raw sewage samples. Furthermore, when a significant number of viruses is expected in a sample, the enumeration of the adsorbed viruses could be obtained directly with cell culture without a need for an elution step (Papaventsis et al., 2005). The recovery rate using negatively charged membrane depends on the water matrix and virus type (Hamza et al., 2009; Haramoto et al., 2009; Katayama et al., 2002; Victoria et al., 2009). Hamza et al. (2009) reported recovery rates from 21.3% to 100% for JCPyV, AdV, Echo 11, and NoV from river water.

Positively charged filters are able to enrich viruses without prior conditioning of the sample examples of positively charged filters include Virosorb 1MDS and more economic alternatives, such as NanoCeram filters (approximately 20% of the cost of the 1MDS filter), glass wool, and the ViroCap capsule filter. The recovery efficiency from the positively charged membrane is influenced by pH, the type of water, organic compounds, and turbidity (Enriquez and Gerba, 1995; Katayama et al., 2002; Lukasik et al., 2000; Sobsey and Glass, 1984). Different studies have evaluated the 1MDS filter and showed that the filter is efficient to recover viruses from different types of water (Dahling, 2002; Karim et al., 2009). The NanoCeram filter is equivalent in performance to the 1MDS (Ikner et al., 2011; Karim et al., 2009) and has been used for virus recovery from surface water, drinking water and wastewater (Prevost et al., 2015; Qiu et al., 2015; Ye et al., 2012). Also, the Nanoceram filter is incorporated in USEPA Method 1615 to study the occurrence of EV and NoV in water by cell culture and RT-qPCR. Francy et al. (2013) evaluated NanoCeram, glass wool, and ViroCap for the concentration of viruses; all methods revealed relatively low recovery rates (0–14.5%) for human viruses. However, high virus retention (˜ 99%) by NanoCeram has been demonstrated (Ikner et al., 2011). Glass wool has been used in many laboratories to concentrate enteric viruses from environmental water samples (Ehlers et al., 2005; Lambertini et al., 2008; van Heerden et al., 2005). Previous studies reported recoveries of 72% and 75% for PV from drinking water and sea water, respectively (Vilaginès et al., 1997). Another study by Lambertini et al. (2008) showed high recoveries of 98% for PV, 30% for NoV and 28% for AdV from tap water. The main advantages of these filters lie in the large volumes that can be used before clogging and without pre-conditioning. The only pre-treatment necessary is dechlorination of drinking water.

Virus concentration by entrapment involves ultrafiltration and ultracentrifugation techniques. Ultrafiltration can be done by passing the water sample through capillaries, membranes, or hollow fibers, with cut off levels of 30–100 kDa. Most ultrafilters employ tangential flow filtration (TFF). Prefiltration of water samples is required to remove suspended solids to limit filter clogging. Ultrafiltration procedures have been applied to concentrate viruses from different types of water and sewage samples (Divizia et al., 1989; Francy et al., 2013; Grassi et al., 2010; Hewitt et al., 2007; Kahler et al., 2015; Rajal et al., 2007). Principally, ultrafiltration requires no preconditioning of the sample so a wide range of viruses can be recovered. The method can be used as a reconcentration step as well. Ultracentrifugation separates viral particles based on both their density and size. Ultracentrifugation can be used directly to concentrate viruses from small volume of sewage or as a re-concentration step in case of surface water because limited volumes of water can be processed (Albinana-Gimenez et al., 2006; Nordgren et al., 2008). Ultracentrifugation is not widely used in viral analysis of environmental water samples due to the high capital cost and the portability of the instrument that limit its direct use at sampling sites. A secondary concentration step may be required to reduce the volume of virus concentrate to a manageable volume for the detection method. Common reconcentration protocols include PEG precipitation, organic flocculation, ultrafiltration and ultracentrifugation.

Animal cell culture is the gold standard method for human virus

Table 1
Advantages and disadvantages of molecular methods used in environmental virology.

Method	Advantage	Disadvantage
PCR or real-time PCR	Rapid; highly sensitive and specific.	Sensitive to the inhibitors; Unable to assess infectivity.
Long target region (LTR) PCR	Rapid; specific; may assess the genome integrity.	Insufficient to completely assess viral infectivity; may have lower sensitivity.
ICC-PCR	More rapid and sensitive than cell culture alone; detect infectious viruses; less affected by PCR inhibitors.	Costly; not appropriate for non-culturable viruses; background contamination of non-infectious viruses is possible.
IMS-PCR	Reduces the possibility of co-concentration of PCR inhibitors; detection of intact viral particles.	Affected by complex matrices of the sample; costly; may not target all strains of viruses.
Enzymatic treatment or dye treatment	Rapid assess of damaged viral capsid proteins; no need for cell culture.	Uses capsid integrity as the criterion of infectivity; needs case by case optimization; high concentration of dye or enzyme may affect the reaction.
Digital PCR	Less affected by PCR inhibitors; less competition of DNA background; higher precision; no standard curve.	Expensive instrumentation and reagents; limited reaction volume; low dynamic range; low throughput; technical complex compared to qPCR.
NASBA	Less affected by inhibitors in the sample; with comparable sensitivity or higher sensitivity than PCR; selective amplification of RNA in the presence of DNA background; no need for thermal cycler.	Contains thermolabile enzyme so the reaction cannot exceed 42 °C; limited size of amplicon; affected by the integrity of RNA.
Microarray	Simultaneous detection of multiple pathogen targets.	Expensive; lower sensitivity than PCR; sensitive to variation in hybridization temperature; requires complex analysis; lack of control over the pool of analyzed transcripts.
Metagenomics	No need for culturing or cloning prior to sequence analysis; relatively unbiased; provides detailed information on microbial diversity.	Expensive; complex data analysis; cannot study less abundant genomes; methods still under development.

detection and quantification via observation of cytopathic effects. However, conventional cell culture is expensive, laborious, and not all enteric viruses propagate in cell culture; thus, it cannot be used in all applications. Currently the detection of human viruses in aquatic environment is primarily based on molecular techniques. Herein, this review discusses the most applied molecular tools in environmental virology and the possible alternatives to improve the virus detection in water in terms of sensitivity, diversity and selective detection of infectious viruses. Table 1 summarizes the advantages and disadvantages of these approaches.

2. Polymerase chain reaction

Since the early 1990s, PCR has been considered the reliable method to detect viral nucleic acids in environmental samples due to the high specificity and sensitivity. The sensitivity of PCR has been demonstrated to be comparable or superior to cell culture (Hamza et al., 2011b; Lee and Jeong, 2004). A drawback of PCR compared to cell culture is that nucleic acids from non-infectious virus may be detected. Real-Time PCR, also commonly called quantitative PCR (qPCR), represented the most significant advance in virus detection in water environment. QPCR provides relative quantification; for absolute quantification, a standard curve is required from an absolute standard with known concentration of target nucleic acid. A qPCR assay can be used to detect amplified DNA using SYBR Green dye or for specific target detection by using an oligonucleotide hydrolysis probe, such as a Taqman probe, Fluorescence Resonance Energy Transfer (FRET), or a molecular beacon. Based on the primers/probes used, qPCR can be used not only for detection and quantification but also for genotyping. Standardized parameters, called minimum information for publication of quantitative real time PCR (the MIQE guidelines) should be reported for publication of qPCR experiments (Bustin et al., 2009). qPCR has other advantages as well. For example, in qPCR both the amplification and detection of the target gene are combined in one reaction tube, which in turn reduces the risk of carry over contamination. Like conventional PCR, qPCR uses oligonucleotide primers it can be also used in a multiplex format for amplification of several target sequences in one tube (Fout et al., 2003).

Environmental samples contain a large variety of organic and inorganic contaminants that are known to inhibit the DNA polymerase directly or indirectly leading to decreased sensitivity or PCR inhibition. For instance, sewage samples may contain common PCR inhibitors such as calcium ions, humic acids, metal ions, polyphenols, fats and proteins. High concentration of calcium inhibits DNA polymerase or reverse trancriptase activity via competitive binding with the DNA polymerase instead of magnesium ions. Low concentration of humic acids interact with the nucleic acids and the enzymes preventing the PCR (Sutlovic et al., 2005, 2008). In addition, phenols may cross-link RNA under oxidizing conditions, change the chemical properties of the nucleic acids and hamper the RNA extraction (Schrader et al., 2012).

PCR inhibitors can be reduced or removed using different strategies that include guanidinium thiocyanate extraction, phenol–chloroform extraction, chemical flocculation using multivalent cations and ultrafiltration. These methods are reported to be more efficient than gel filtration (Braid et al., 2003). In addition, some adsorbents or column chromatography such as Sephacryl s-400, Sephadex G-100 and G-200, and polymeric adsorbents DAX-8 and polyvinylpolypyrrolidone can significantly reduce the co-purification of PCR inhibitors (Abbaszadegan et al., 1993; Hale et al., 1996; Miller et al., 1999; Schriewer et al., 2011; Zhou et al., 1996). Also, using additives directly to the PCR reaction such as T4 bacteriophage gene 32 protein and bovine serum albumin are known to be more effective against different PCR inhibitors (Kreader, 1996). Dilution of extracted nucleic acid will also dilute the inhibitors but it is accompanied by decrease in sensitivity (Brooks et al., 2005). In order to investigate the presence of inhibitory substances, PCR inhibition control reaction should be used which consists of known amount nucleic acid or microorganism and analyzed in parallel to the target sequence. The general properties of PCR inhibitors, their removal and mechanisms of action have been previously reviewed by Schrader et al. (2012).

Numerous studies have been conducted using PCR/qPCR in different environmental water samples and waterborne viral outbreak investigations (Divizia et al., 2004; Hoebe et al., 2004; Maunula et al., 2004, 2009; Scarcella et al., 2009; Sinclair et al., 2009). Although qPCR provides the high sensitivity and specificity of detection, the efficiency of qPCR can be negatively affected by the presence of PCR inhibitors generally found in water concentrate leading to false negative results or underestimation of vial concentration (Hamza et al., 2009). In addition, qPCR may overestimate the presence of viruses because of the co-detection of nucleic acids from both viable and non-viable viruses/agents. Previous investigations have found higher quantities of AdV and EV by qPCR compared with conventional cell culture (Choi and Jiang, 2005; Hamza et al., 2011b). However, to estimate the public health risk associated with water contamination, the detection of infectious viruses is most important. Accordingly, some modifications have been proposed to the standard PCR/qPCR to overcome the critical issues associated

with inability to discriminate between infectious and nonviable viruses, as discussed below.

2.1. Long target region (LTR) PCR

PCR of a long target region has been proposed to investigate viral infectivity. This approach is based on the fact that an intact viral genome is necessary for the virus to remain infectious, and a long PCR region would be expected to be more representative of an intact viral genome (Allain et al., 2006; Li et al., 2002b; Simonet and Gantzer, 2006a; Wolf et al., 2009). HAV infectivity was previously assessed using this protocol after chlorine treatment; results showed that the HAV genome has different degrees of sensitivity to chlorine, depending on the position of chlorine action – 5`NTR and 3`NTR were more sensitive to chlorine (Li et al., 2002a). LTR-RT-PCR targets were used to estimate the effect of chlorine dioxide on PV (Simonet and Gantzer, 2006a). The results revealed no linear relationship between the degradation of the viral genome and the size of the target gene. Rapid inactivation of PV was found as estimated by cell culture compared to $> 3\ \log_{10}$ reduction of 5′UTR fragments. Simonet and Gantzer (2006b) investigated the ability of LTR-RT-PCR to identify infectious PV1 and MS2 after UV exposure. A linear correlation was found between RNA fragment size and degradation rate. The integrity of murine norovirus (MNV) and human norovirus was examined using the duplex RT-qPCR for MNV-1 for the simultaneous detection after UV and heat inactivation (Wolf et al., 2009). The authors found that although short target PCR was not affected by the inactivation process, the decrease in LTR correlated with the increase of UV. It was also found that the reduction in qPCR signal correlated with amplicon length and UV inactivation dose. Detection of inactivated ΦX174 by a long amplicon of nearly the complete genome had similar results compared with plaque assay (Ho et al., 2016).

Virus inactivation does not necessitate genome damage, therefore LTR is insufficient to completely assess viral infectivity. Also, LTR may reduce the sensitivity of PCR; thus, the feasibility of this approach as a surrogate marker for viral infectivity is limited.

2.2. Integrated cell culture PCR (ICC-PCR)

An integrated cell culture/PCR method (ICC-PCR) allows fast and sensitive detection of infectious viruses compared with the use of cell culture alone. ICC-PCR has been developed to overcome the limitation of sole use of PCR and cell culture. Although cell culture is time consuming and has less sensitivity and specificity than PCR, it detects only infectious viruses. Sample inoculation in cell culture may also eliminate or minimize the inhibitory effects of environmental samples prior to PCR. The protocol has been first proposed to be used in environmental virology by Murrin and Slade (1997), Reynolds et al. (1996). ICC-PCR has been used to detect many enteric viruses including: AdV, RoV, HEV, EV and AstV in environmental samples or to investigate the efficiency of viral disinfection (Balkin and Margolin, 2010; Chapron et al., 2000; Dong et al., 2010; Hamza et al., 2011b; Lee and Jeong, 2004; Lee et al., 2005).

ICC-PCR has proved to be more sensitive than conventional cell culture to discriminate between infectious and nonviable PV following chlorine disinfection, and minimized the risk of false negative results caused by testing only one passage of cell culture (Blackmer et al., 2000). ICC-PCR has been evaluated by Lee et al. (2005) for the simultaneous detection of EV and AdV and compared with conventional cell culture. Approximately 67% of surface water and 46% of tap water exhibited CPE by conventional cell culture; however, by using ICC-PCR the detection rate was increased to 77% and 58% for surface water and tap water, respectively.

Li and coworkers proposed the use of MA104 cells combined with qPCR to detect infectious RoV (Li et al., 2010). The limit of detection of ICC-PCR after two days of incubation was 0.2 PFU/ml. To assess the protocol, the authors used heat-inactivated RoV and compared the results obtained by ICC-qPCR to qPCR alone. Although the qPCR results did not change after heat inactivation of RoV, the amount of RoV decreased after 1 min inactivation when estimated by ICC-qPCR (Li et al., 2010). In field samples, RoV were detected in 42% of the samples using ICC-qPCR, whereas using plaque assay or qPCR alone RoV could be detected in 21% and 12% of samples, respectively (Li et al., 2010). Recently, ICC-qPCR was also used to investigate the resistance of type 2 AdV in disinfection studies (Ryu et al., 2015). The virus concentration obtained by the conventional cell culture and ICC-qPCR was highly correlated ($R^2 = 0.96$), indicating that ICC-qPCR is an alternative approach to quantify AdV in disinfection assays.

DNA viruses that do not replicate well or are slow growing in a cell culture system can be monitored via the detection of late genes of replication in infected cells by targeting virus-specific mRNA. RT-PCR of AdV mRNA, which are difficult to propagate in cell culture, was described to discriminate between infectious and nonviable adenovirus (Ko et al., 2003). Six hours post-infection, HAdV-2 mRNA was detected and HAdV-41 could be detected after one day of A549 cells infection. In a later study, Ko et al. (2005) used the same approach coupled with qPCR to investigate the resistance of enteric adenovirus to UV disinfection. Detection of AdV-41 mRNA showed that the virus was more resistant to UV disinfection compared to other studies in which traditional cell culture method was used. Detection of (–)RNA as a replicative intermediate during replication of (+)RNA virus as a sign of infectivity has been proposed to discriminate between infectious and nonviable viruses by Jiang et al. (2004). The approach was used to check the infectivity of HAV in water samples and showed sensitivity of one TCID50 per flask during four-day incubation, revealing sensitive and reliable detection of infectious HAV. Finally, alternative approaches to ICC-PCR have been proposed, including direct DNA extraction on infected cells and the application of quantitative PCR (Ogorzaly et al., 2013b). These additions significantly increased the speed and accuracy of detection using ICC-PCR (Ogorzaly et al., 2013b).

ICC-PCR has some drawbacks. Even though ICC-PCR reduces the time for detection of infectious viruses compared to traditional cell culture and reduces PCR inhibition, the assay is costly and the detection of background contamination of non-infectious viruses is possible. Also, the primer sets used to detect indigenous enteric viruses isolated from environmental samples by ICC-PCR may reduce the reliability of the assay (Lee and Jeong, 2004). Finally, some viruses such as human NoV don't have readily-available cell lines for the propagation. Methods to differentiate infectious NoV have been previously reviewed (Knight et al., 2013). Although Straub and co-workers have proposed propagation of HuNoV on 3D intestinal epithelial cells of Int-407 and CaCo-2 (Straub et al., 2011, 2007, 2013), other independent attempts have failed to replicate norovirus on the 3D organoid cell culture models (Papafragkou et al., 2014; Takanashi et al., 2014), highlighting the complexity of developing a reproducible in vitro cell culture system for HuNoV. A recent breakthrough described by Ettayebi et al. (2016) used stem-cell-derived intestinal enteroids as an in vitro culture approach for NoV suggests the potential for future development in this area.

2.3. Immunomagnetic separation prior to PCR (IMS-PCR)

The recovery of enteric viruses from water samples has been achieved by using immunomagnetic separation (Casas and Sunen, 2002; Myrmel et al., 2000). The method uses antibody-coated paramagnetic beads to bind the specific surface antigen of a particular pathogen to facilitate its concentration. The assay concentrates viral particles and reduces the possibility of co-concentration of PCR inhibitors.

The method was previously combined with qPCR to detect HAV, RoV, HAdV, TTV and human polyomavirus (HPyV) in environmental samples (Abd El Galil et al., 2005; Haramoto et al., 2010; Yang et al., 2011). Discrimination between infectious and nonviable viruses relies on the properties of certain viral capsid proteins. Non-infectious viral

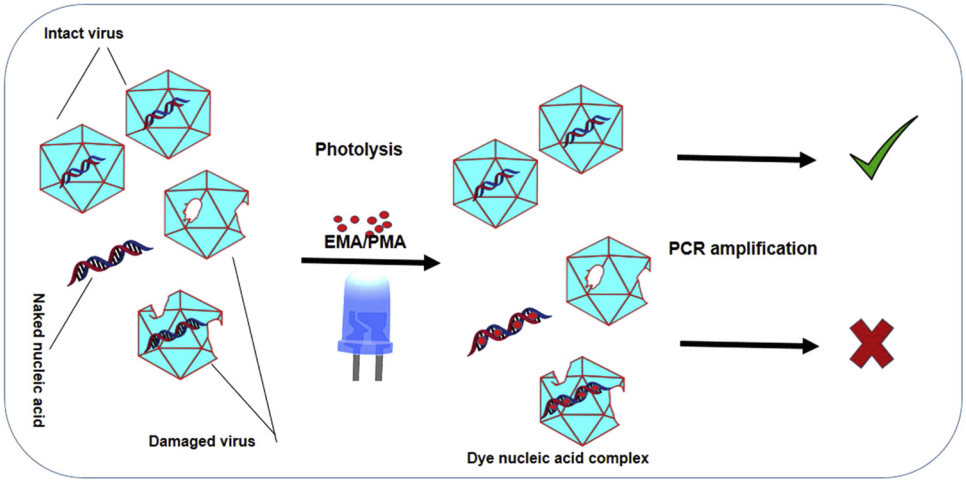

Fig. 1. Mechanism of PMA and EMA treatment prior to PCR amplifications.

particles may have damaged viral capsid proteins, which in turn inhibits the formation of antigen-antibody complexes, leading to negative results in IMS-PCR. IMS-qPCR provided the same sensitivity as conventional plaque assay to detect infectious EV spiked in water samples with a sensitivity of one PFU (Hwang et al., 2007). Another study by Yang et al. (2011) showed that inclusion of an IMS step in qRT-PCR increased the detection sensitivity of rotavirus by one order of magnitude.

The disadvantage of immunological preparation methods is that the binding capacity is affected by complex matrices of the sample, amount of colloidal particles, and water type (Yang et al., 2011). Additionally, the antibody used may not target all strains of viruses or all enteric viruses so a specific assay is needed for each virus.

Similar assays of potential interest have also been developed. For example, a previous study developed antigen-capture PCR for the detection of HAV in environmental samples (Deng et al., 1994). Good correlation was observed between antigen-capture PCR and cell culture quantification (Deng et al., 1994). Similarly, an immunocapture approach coupled with qPCR was previously developed to quantitatively detect adenoviruses in environmental samples (Ogorzaly et al., 2013a). This assay was able to quantitatively detect structurally intact adenovirus with enhanced sensitively compared to ELISA (Ogorzaly et al., 2013a).

2.4. Enzymatic treatment of water samples prior to PCR

Enzymatic treatments have previously been developed to adapt PCR to detect infectious viruses. The treatment process includes proteases and RNase/DNase prior to PCR to remove DNA or RNA from damaged viral particles. The loss of viral capsid protein integrity results in viral inactivation while the viral DNA or RNA may remain detectable by PCR. A compromised viral capsid is more sensitive to protease degradation than the intact viral capsid and consequently releases the viral nucleic acid, which are then more susceptible to nucleases than capsid-enclosed nucleic acids. Enzymatic treatment has also been used to assess the HAV, FCV, and PV inactivation by heat, ultraviolet light, and sodium hypochlorite (Nuanualsuwan and Cliver, 2002). Although enzymatic treatment was successful to detect inactivation of viruses using heat, UV irradiation, and chlorine, the nucleic acids of viruses inactivated by incubation at 37 °C were still protected by the capsid (Nuanualsuwan and Cliver, 2003). Enzymatic treatment was used to study the infectivity of MNV after thermal inactivation at 80 °C (Baert et al., 2008). The study showed that thermal inactivation had a much stronger effect on the infectivity than the integrity of the viral genome. Accordingly, > 6 $\log_{10}$ reductions were estimated by plaque assay, whereas 9 $\log_{10}$ RNA were detected by qRT-PCR. Viral stability in environmental samples has been investigated using enzymatic treatment (Bofill-Mas et al., 2006). DNase treatment of viral suspension revealed that the T_{99} (time for 99% inactivation) of HAdV and JCPyV was ˜126 and 121 days, respectively. Without treatment, the T_{99} was 132.3 days for HAdV and 127.3 days for JCPyV. The effectiveness of enzymatic treatment was assessed using MS2 bacteriophage in treated wastewater (Unnithan et al., 2015). It was proposed that the use of RNase A at an appropriate concentration could be as effective as using both proteases and RNase. Enzymatic treatment coupled with PCR was used to quantify the infectivity of MS2 following heat exposure, singlet oxygen, and UV radiation using primer sets that cover the entire coding region and results revealed that qPCR overestimated the infectivity (Pecson et al., 2009). Enzymatic treatment reduced the qPCR signal by > 5 $\log_{10}$ and the degree of inactivation depended on the type of inactivation treatment. Pecson and co-workers pointed out that no complete concordance between infectivity and PCR assay could be found.

Enzymatic treatment could be an alternative approach to overcome the disadvantage of traditional cell culture assay. Nevertheless, PCR inhibitors present in environmental samples can reduce the efficacy of the assay. In addition, the correlation between infectivity and PCR results after enzymatic treatment depends on the mechanisms of inactivation, type of treatment, and the concentration of the enzymes.

2.5. Intercalating dye treatment of water samples prior to PCR

A promising strategy for the detection of viable pathogens is by using dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA) to intercalate between the bases of free nucleic acids. These dyes function via a photo-inducible azide group, which covalently cross-links to DNA leading to inhibition of PCR amplification (Nocker and Camper, 2006). Intact viruses will not allow EMA or PMA to enter, while damaged virus capsids allow the dye to interact with viral nucleic acids through capsid intrusion. Fig. 1 shows the mechanism of dye treatment.

Dye treatment has recently been evaluated to identify infectious viruses in environmental matrices. The survival of avian influenza in water over 56 days at 21 °C and 37 °C has been investigated by EMA-qPCR, cell culture, and qPCR (Graiver et al., 2010). The viral concentration obtained by EMA-RT-PCR was higher than that obtained by cell culture titration and no significant difference was found between the RT-PCR and EMA-RT-PCR. This could be because the dye did not effectively interact with viral RNA of non-infectious virus or with extracted RNA of avian influenza. Another study attempted to distinguish between infectious and non-infectious poliovirus after heat treatment at 45 °C, 55 °C, 65 °C, and 75 °C after EMA treatment (Kim et al., 2011);

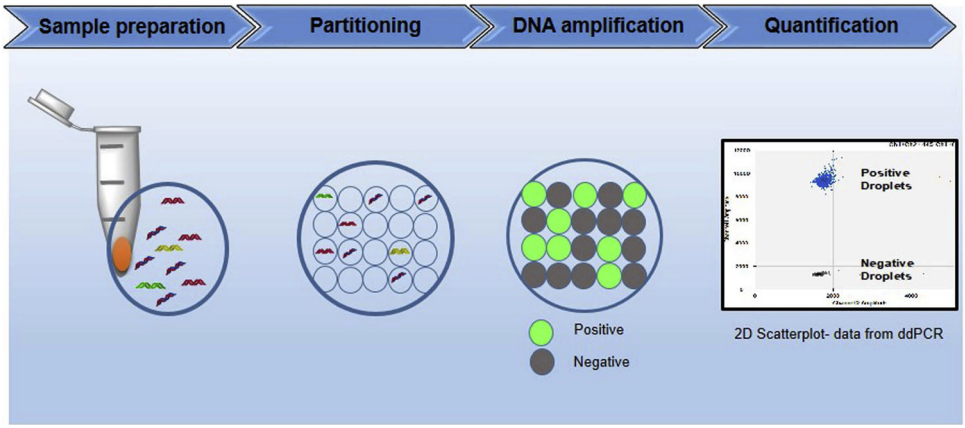

Fig. 2. The key steps of digital PCR analysis.

EMA-RT-PCR was comparable with quantification by plaque assay. Kim and co-workers have used different concentrations of EMA to evaluate infectious viruses, and observed a 4-log reduction in viral RNA concentration after adding 10 μg/ml-EMA. PMA treatment was also used prior to RT-PCR to differentiate between potentially infectious and noninfectious viruses, including coxsackievirus B5, echovirus 7, PV, and NoV after thermal inactivation or chlorination (Parshionikar et al., 2010). The PMA-RT-PCR treatment prevented the detection of inactivated viruses by heat treatment at 72 °C, 37 °C and hypochlorite; however, at 19 °C the assay was unable to distinguish between infectious and non-infectious enteroviruses and NoV and all PMA-RT-PCR results were positive (Parshionikar et al., 2010). In order to test interference due to environmental water matrices with the PMA-RT-PCR assay, the authors used PMA-RT-PCR to examine the infectivity of PV seeded in environmental water samples and was able to distinguish between infectious and noninfectious PV (Parshionikar et al., 2010). The efficacy of PMA-qPCR to inhibit PCR amplification from non-infectious T4 bacteriophage was compared to plaque assay and qPCR (Fittipaldi et al., 2010). PMA treatment was not effective to discriminate between infectious and non-infectious bacteriophage T4 after heat inactivation (85 °C) and protease treatment; however, under heat inactivation at 110 °C, PMA-qPCR allowed the differentiation of the infectious from non-infectious bacteriophage, due to effective binding of PMA to bacteriophage T4 DNA indicating capsid damage (Fittipaldi et al., 2010). EMA/PMA-qPCR of inactivated RoV, PV, murine norovirus, AdV, and øX174 was presented by Leifels et al. (2015). Dye treatment of UV- and heat inactivated viruses did not correlate with the results of the cell culture, whereas EMA/PMA-qPCR of viruses inactivated by chlorine treatment was consistent with cell culture. Leifels et al. (2015) suggested that dye treatment approaches should be tested for each virus separately because different viruses could have different degrees or mechanisms of inactivation.

In addition, to improve the efficiency of dye treatment for selective detection of infectious viruses some detergents such as Triton X-100 and sodium lauroyl sarcosinate have been suggested (Fuster et al., 2016; Lee et al., 2018). Also, a new version of PMA named PMAxx has been design by Biotium to be more selective than PMA and inhibit PCR amplification of dead cells. PMAxx has been recently used to distinguish between infectious and nonviable NoV in sewage and shellfish (Randazzo et al., 2018); however, the assay reduced the signal of thermally inactivated norovirus and did not completely remove it, still resulting in an overestimation of infectivity.

There is discrepancy between published reports of intercalating dye treatments to distinguish between infectious and non-infectious viruses. Therefore, the method should be adapted for each virus separately. Although the assay still may overestimate the infectivity, it is potentially more representative than conventional PCR/qPCR without dye treatment. However, optimization of dye concentration, incubation time, distance to the light source and the light source alter the effectiveness of the assay.

3. Digital PCR

Digital PCR (dPCR) is a next-generation PCR for the sensitive and specific detection and quantification of nucleic acid without using a standard curve, enabling an absolute quantification with much higher precision compared to qPCR. dPCR begins with partitioning the reaction mix into hundreds to thousands of independent PCR sub-reactions and the partitions are then thermally cycled to end-point. The number of positive and negative sub-reactions are then read; this proportion is directly proportional to the total number of molecules present in the original sample, and the target concentration is calculated using binomial Poisson statistics (Dube et al., 2008; Pinheiro et al., 2012). In 2006, the first commercial chip-based digital PCR was offered by Fluidigm and the first commercial digital droplet PCR was launched in 2011 by QuantaLife. The key steps of digital PCR analysis are illustrated in Fig. 2.

Unlike qPCR, the signals of dPCR are measured after the complete PCR amplification. dPCR provides a binary output since each portion is negative or positive and the quantification is independent of the PCR efficiency; therefore, digital PCR reduces the influence of PCR inhibitors (Hoshino and Inagaki, 2012). The distribution of the reactions also reduces competition due to background DNA.

The first dPCR quantification of a virus in water sample was completed using a one-step RT droplet digital PCR (RT-ddPCR)-based absolute quantification of RoV in surface water (Racki et al., 2014). The results of RT-ddPCR were superior to RT-qPCR in quantification performance with higher precision and repeatability of RoV at the low concentrations expected in water samples. Additionally, RT-ddPCR showed better tolerance to PCR inhibitors from water matrices. Accurate quantification of AdV in Japanese river water samples using microfluidic dPCR was investigated and compared to qPCR and MPN-PCR (Kishida et al., 2014). However, the precision and sensitivity of dPCR was superior to qPCR and MPN-PCR as the detection frequency of dPCR was moderately higher than those of qPCR. Accordingly, dPCR was judged to be suitable for quantitative microbial risk assessment because accurate and sensitive data are required to increase the precision of the assessment. (Kishida et al., 2014). A recent study comparing RT-qPCR and RT-ddPCR to quantify norovirus in oysters found greater precision with comparable limits of quantification for RT-ddPCR (Persson et al., 2018). Another recent study found greater reproducibility and sensitivity to detect Sapovirus in RT-ddPCR than RT-qPCR (Varela et al., 2018). The performance of RT-qPCR and RT-dPCR for norovirus for risk assessment was comparable (Monteiro and Santos, 2017). Coudray-Meunier et al. (2015) compared the performance of microfluidic digital RT-PCR to RT-qPCR for the detection of NoV and HAV spiked in bottled

water and lettuce. The recovery of spiked viruses as measured by dPCR was higher than that measured by qPCR. The study also found that microfluidic dPCR was more robust in the presence of PCR inhibitors compared to qPCR. A similar study also found than dPCR reduced PCR inhibition to detect HAV and NoV in berries (Fraisse et al., 2017). The study highlighted a novel application of microfluidic RT-dPCR to quantify viral loads in water at a cost estimated to be half that for RT-qPCR.

Finally, ddPCR has been used to quantify human pathogenic viruses and markers of fecal contaminations in the stormwater discharges in California demonstrating its successful application under realistic conditions; NoV was the most abundant virus detected (96%), AdV was detected in 22% of samples and EV was not found in any stormwater discharge (Steele et al., 2018).

Indeed, an important advantage of dPCR over qPCR for its application in environmental virology is the tolerance to inhibitors. Since the method has rarely been used to quantify enteric viruses in water, further evaluations are needed under different conditions of water quality.

4. Nucleic acid sequence-based amplification

Nucleic acid sequence-based amplification (NASBA) is an alternative method of RT-PCR that has been developed to detect RNA using isothermal amplification. NASBA has a shorter time (˜100 min) compared to PCR and employs three different enzymes; avian myeloblastosis virus reverse transcriptase (AMV RT), RNase H, and T7 RNA polymerase as shown in Fig. 3. Both dNTPs and NTPs are involved in the reaction. The AMV RT enzyme synthesizes the complementary DNA strand of a given RNA using one of the primers included in the reaction. RNAse H removes the RNA template from RNA-DNA hybrid to allow binding of the second primer to cDNA, producing double stranded DNA by RT enzyme. Afterward, the T7 polymerase transcribes the double stranded DNA to copy the RNA target.

In contrast to clinical applications, few studies have employed NASBA for the detection of enteric viruses in environmental samples. NASBA techniques were established to detect the vp2 gene of HAV (Jean et al., 2001). In a pure virus suspension, the detection limit of NASBA was 2PFU. In artificially contaminated wastewater samples, the authors stated that the specificity of the NASBA system and its ability to detect HAV could be achieved without any interference in complex samples. However, it should be noted that Jean et al. (2001) only used 5 µl of wastewater samples heated to 100 °C to lyse the viral particles prior to NASBA, which is too small of a volume to be used in naturally contaminated samples of which large volume should be concentrated and might co-concentrate NASBA inhibitors as well (Rutjes et al., 2005).

Multiplex NASBA was also evaluated for the simultaneous identification of HAV and RoV (Jean et al., 2002b). The assay used pure viral suspension and two sets of primers specific for both viruses were used, respectively. It was modified afterward for rapid detection of RoV using NASBA combined with ELISA (Jean et al., 2002a) and primers targeting the VP7 region and biotinylated 16-uracil triphosphate were used to produce biotinylated RNA amplicons which were hybridized with specific immobilized aminolinked DNA probe on microtiter plate. Using this system, the assay detection limit was 0.2 PFU/ml and 15 PFU for RoV seeded in ultrapure water and sewage treatment effluent, respectively, in 6 h. The limit of detection of this study is almost 10 times more sensitive than that obtained by the conventional NASBA used for HAV by the same group (Jean et al., 2001). Accordingly, the authors demonstrated that the combination of microplate hybridization with NASBA could improve the sensitivity and specificity and can allow the detection of simultaneous samples. Multiplex NASBA showed lower signal compared to monoplex NASBA, both formats showed similar detection limits for HAV and RoV. The detection limits of RoV and HAV were 40 PFU/ml and 400 PFU/ml, respectively. While the assay explored the potential of multiplex NASBA as a reliable approach for the simultaneous detection of HAV and RoV, further evaluations are required for its application to detect viral contamination in environmental water samples (Jean et al., 2002a).

Rutjies and co-workers developed a real-time NoV NASBA targeting part of the RNA-dependent RNA polymerase (RdRp) gene for NoV detection in surface water (Rutjes et al., 2006). NASBA results were compared with RT-PCR and found that the NASBA assay was more resistant to RT-PCR inhibitors. In order to examine whether NASBA

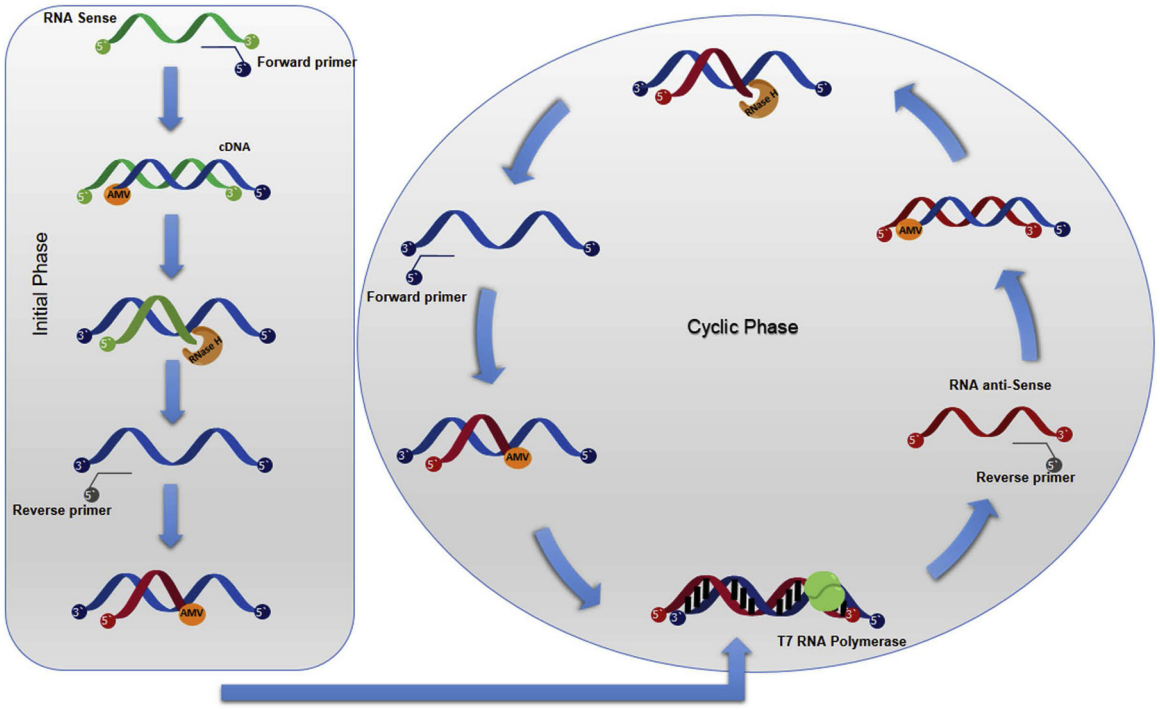

Fig. 3. Scheme for the amplification of RNA by the NASBA, showing the cycling and non-cycling phases. Adapted from (Honsvall and Robertson, 2017) with kind permission from Elsevier.

affected by inhibitory substances in the RNA samples, the authors analyzed RNA samples derived from 12.5 µl and 125 µl river water concentrates. The detection of NoV RNA by RT-PCR in 12.5 µl and 125 µl samples revealed higher viral concentration in most of 12.5 µl samples. Whereas, by using NASBA in 125 µl concentrates a higher quantity of NoV was found, indicating the reduced influence of inhibitory effects for the NASBA assay. In contrast, Abd El Galil et al. (2005) used real time NASBA to detect different dilutions of HAV spiked in surface water and found that the fluorescent signals did not change, indicating an intensive inhibitory effect.

Generally, NASAB assays are more complex compared to PCR, since three enzymes are required in the reaction. Both PCR and NASBA require sample pretreatment, such as the enrichment step of environmental samples prior to virus detection step, but NASBA is less affected by inhibitors which could be co-concentrated with viruses. The assay is isothermal which means that direct heat shock, which could be used instead of nucleic acid extraction in PCR, is not applicable for NASBA. Therefore, further developments are needed before NASBA can be reliably deployed in environmental virology as a routine assay.

5. DNA microarray

DNA microarrays were developed in the 1990s. In DNA microarray assays, complementary oligonucleotide probes are used to detect target sequences in the same sample. Microarrays are a high-throughput screening tool capable of detecting over 10,000 targets during a single test. Commercially available microarray chips are produced with different manufacturing methods and features. To increase the power of identification, the probes are primarily immobilized on a membrane with a line blot format, solid surfaces, or bound to microbeads (Chizhikov et al., 2002; Pagotto et al., 2008; Vinje and Koopmans, 2000). An example of DNA microarray is presented in Fig. 4. DNA microarrays have been primarily used in a variety of clinical applications, gene expression and environmental monitoring (Ayodeji et al., 2009; Brinkman and Fout, 2009; Chizhikov et al., 2002; Jaaskelainen and Maunula, 2006; Miller and Tang, 2009; Wang et al., 2002). Utilization of microarrays for the detection of enteric viruses in the aquatic environment has been limited; however, few reports have evaluated the reliability of microarray to environmental application and were primarily used for simultaneous detection of multiple pathogens and indicators, as discussed below.

A generic microarray format was evaluated as a tool for simultaneous identification of different NoV strains seeded in tap and river water samples (Brinkman and Fout, 2009). The assay was successful in genotyping of NoV spiked in water samples. This showed the feasibility of microarray for genotyping norovirus in water matrices.

A DNA microarray capable of detecting more than 100 species of enteric viruses was developed and validated by using 10 enteric virus species (Martinez et al., 2015). The assay could detect 1×10^3 virus particles of HAdV, HAstV and RoV-A. Although the assay showed good performance for the detection of calicivirus and RoV-A, a lower sensitivity was found for HAdV and HEV. In addition, discrepancies in the detection of mixed infections were observed as verified by RT-PCR of the tested viruses. Furthermore, the study did not evaluate the utility of the assay in environmental samples, instead they used purified lab strains and tested small volume clinical samples.

The use of a microarray for the multiplex detection and genotyping of NoV by hybridization of the PCR product to an oligonucleotide array called NoroChip was developed (Pagotto et al., 2008). The genotyping capability of NoroChip was increased via amplification of 917 bp of both the polymerase and capsid regions. Validation of the assay was performed using NoV-positive stool extracts. The NoroChip was able to distinguish between GI from GII NoVs and subtype genogroups. Pagotto and colleagues proposed that NoroChip2.0 is a rapid, accurate and a transferable method for characterization of NoVs isolated from different settings (Pagotto et al., 2008). To monitor NoV outbreaks and determine variation in the circulating NoV strains, the NoroChip v3.0 was developed (Mattison et al., 2011) at Health Canada and validated in seven international partner laboratories to screen over 600 potential interactions in a single reaction using 2.4 kb amplicon. The correct genogroup typing information was obtained in six partner laboratories using hybridization to the NoroChip v3.0. Difficulty in obtaining long and specific amplicons of all circulating noroviruses is a limiting factor for the implementation as typing tool. Another study by Ayodeji et al. (2009) developed an oligonucleotide incorporating 13,000 selected HAV, coxsackeiviruses A and B, NoV GI, NoV GII and RoV strains. The applicability of the array for virus identification was examined using amplicons from multiple HAV and coxsackeiviruses strains in which the

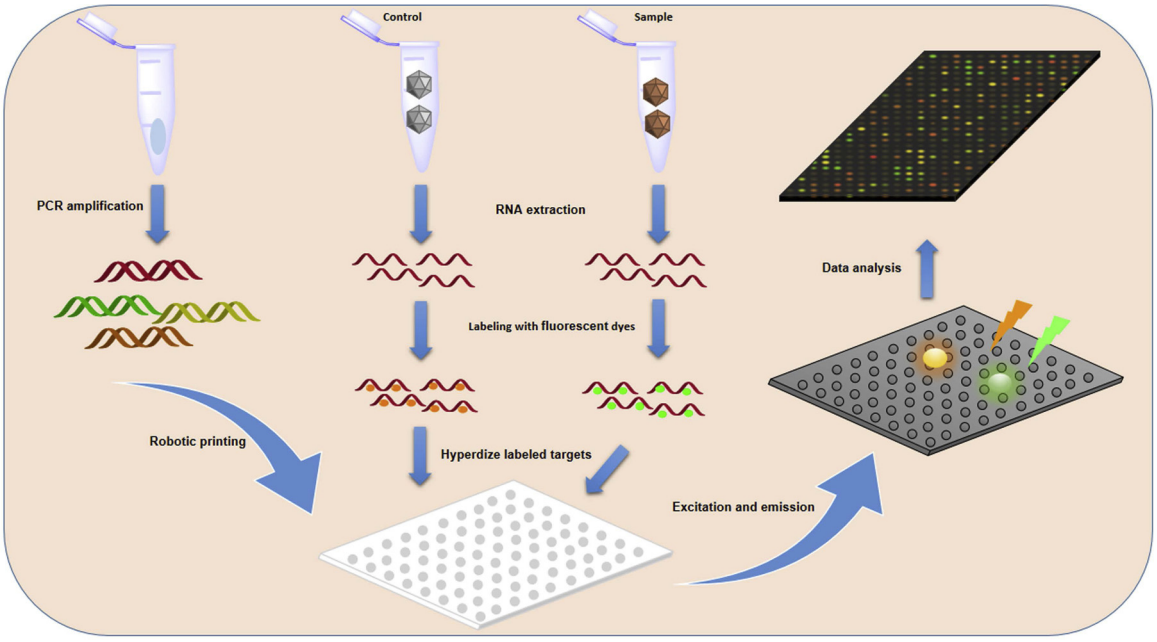

Fig. 4. Primary steps in DNA and RNA target detection and quantification analysis with microarrays. Adapted from (Miller et al., 2015) with kind permission from Elsevier.

biotin was detected using Cy3-labeled streptavidin. The hybridization profile generated was able to discriminate between viral targets at both the genotypes and subgenotypes level.

Wong et al. (2006) designed 780 oligonucleotide probes to detect 25 viruses causing gastroenteritis, and two more probes were added as a quality control. To test the assay, authors used PV and AdV 40 and 41 after culturing on BGM and MA104 cells, respectively. Hybridization signals were obtained and viruses were correctly identified. Wong and coworkers proposed that the developed chip could be used with sewage samples to detect enteric viruses after propagation on cell culture.

Suspension bead arrays have also been used for the high throughput detection of pathogens. The suspension consisted of microspheres 5.6 µm in diameter filled with a relative concentration of an infrared and a red dye to provide a unique spectral identity to each of 100 beads types. Target sequences are amplified using a biotinylated primer and then denatured and hybridized to microspheres tagged with target-specific sequence probes (Dunbar, 2006). Probe-target hybridization is then measured using a streptavidin-bound green fluorophore. A commercial gastrointestinal pathogen panel (GPP kit) is available from Luminex for the simultaneous detection of viruses, bacteria and parasites causing gastroenteritis (Mengelle et al., 2013; Navidad et al., 2013). However, only NoV, RoV and AdV subgenus F could be identified using the GPP kit. Hamza et al. (2014) developed multiplex assay for the simultaneous detection of human enteric viruses in sewage and river water. HAdV, HPyV, EV, RoV, NoVGI and NoVGII were investigated in environmental samples using the Luminex assay and the results compared to monoplex qPCR. The multiplex Luminex assay was as sensitive as qPCR for viral detection in wastewater samples.

Although microarray provides a rapid method for detection genotyping without cloning and sequencing of amplicons and has the flexibility to implement many virus-specific oligonucleotides, its implementation is demanding. The assay requires complex analysis, particularly extensive bioinformatics knowledge is needed to design the assay. The experimental conditions and the design of the probes are the most important parameters to consider. The main factor that affects the applicability of an array in environmental samples is the sensitivity of the assay. In array formats, random amplification is always performed prior to the hybridization to cover broad range of viral panel; however, random amplification could have lower sensitivity. Therefore, the assay is challenged by the ability to identify viral pathogens among other microorganisms in water.

6. Metagenomic analysis

Metagenomics (sometimes called 'shotgun metagenomics') is a powerful tool in which all nucleic acids in a sample are randomly sequenced. Metagenomic approaches include three primary steps: sample preparation, high throughput sequencing, and bioinformatic analysis as shown in Fig. 5.

Several viral concentration and extraction methods have been evaluated in the context of metagenomics, highlighting their impact on viral richness (Hjelmso et al., 2017). The removal of cellular organisms is a crucial step to avoid contaminating viral DNA or RNA with high amounts of non-viral nucleic acids. Viral isolation is typically performed, at least in part, based upon isolating viral sized particles, often called virus-like particles (VLPs). Solids-associated VLPs may be lost during initial sample processing. Sometimes fluorescence microscopy coupled with SYBR gold staining is used to ensure efficient viral concentration (Thurber et al., 2009). To disrupt the bacterial and host cell membranes, samples are treated with chloroform followed by DNase digestion to remove background contamination of DNA. However, this step is selective since enveloped viruses will also lose lipid membranes. In addition, DNase digestion does not completely remove non-viral DNA. RNase could be used when RNA viruses are targeted, but some intact viruses are sensitive to the RNase digestion (Thurber et al., 2009). Once virus particles are purified, several DNA/RNA extraction protocols are available. The specific output of metagenomics sequences can also be increased by using virome specific capturing chip or blood derived antibodies against viral particles (Briese et al., 2015; Oude Munnink et al., 2013). After nucleic acid extraction, the DNA could be amplified using random primers and the whole transcriptome amplification (WTA) kit can be used in the case of RNA viruses to synthesize cDNA (Gensberger and Kostic, 2013; Tomlins et al., 2006). WTA allows the amplification from low concentrations of RNA and produces cDNA library of random overlapped fragments with a universal end sequences. The performance of a WTA kit was evaluated using drinking water samples seeded with bacteria and low concentration of EV RNA. It was found that the kit significantly increased the total number of positives below the detection limit; therefore, WTA amplification increases the target concentration (Parker et al., 2011).

The first viral metagenomic studies were conducted using standard cloning protocols coupled with Sanger sequencing (Angly et al., 2006). However, this may introduce bias because cloning is influenced by DNA properties such as GC%, secondary structure, and some viral sequences are toxic for the bacteria used for cloning (Schoenfeld et al., 2008). In addition, the number of sequences obtained by cloning are limited. Recently, sequence capabilities have exponentially increased compared to Sanger technology via the development of the next generation sequencing technologies such as 454 pyrosequencing (Roche/454), Illuminia/Solexa, and ABI/SOLiD, enabling high-throughput sequencing of unknown viruses and improving viral discovery. Illumina sequencing technology is currently the most widely employed technique. After quality filtering of the reads and excluding contaminating DNA reads, the results are then ready for the taxonomy and functional assignment. Sequence identification is most widely done using BLAST tools. The sensitivity of taxonomic annotation of virus identification has been improved by tBLASTx approach (Aw et al., 2014; Bibby et al., 2011; Vazquez-Castellanos et al., 2014). Assignments can either be directly made on metagenomic reads or reads may be assembled into longer contiguous sequences (contigs). Due to the short length of viral genomes, assembled contigs could represent complete viral genomes. Due to the large number of unidentified viral genomes, many metagenomic

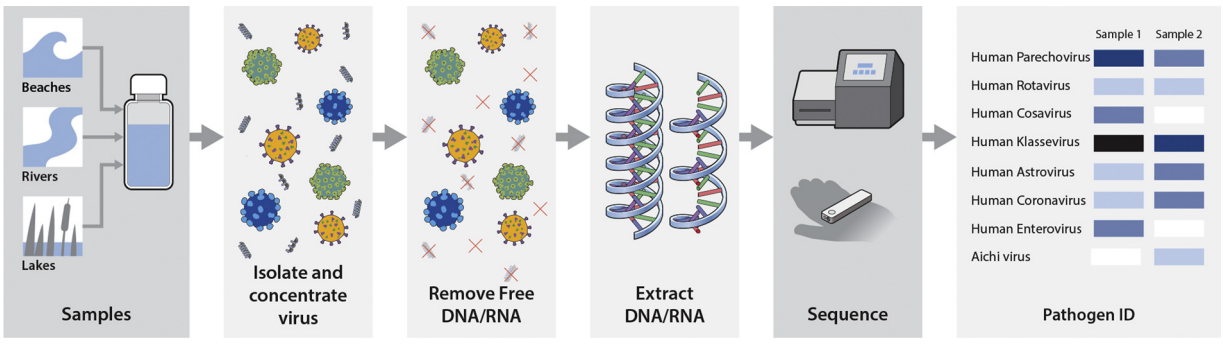

Fig. 5. A diagram of a typical metagenomic approach.

reads and contigs fail assignment using current databases; for example, a viral metagenomic analysis may contain up to 99% unknown sequences (Breitbart et al., 2004a, b; Breitbart et al., 2002; Mokili et al., 2012). Bioinformatics tools able to predict viral sequences are available such as MetaVir (Roux et al., 2011), VirSorter (Roux et al., 2015), and VIROME (Wommack et al., 2012). Comprehensive reviews of bioinformatics tools dedicated to virome analysis (Edwards et al., 2016; Sharma et al., 2015), as well as virome analysis for pathogen identification in environmental samples (Bibby, 2013) have been described elsewhere. Also, most of the assembly program were designed for single genome assembly, therefore many assembly errors could be produced from conserved regions within viral species (Rastrojo and Alcami, 2016). Since the development of next generation sequencing, the number of aquatic metagenomic studies has been growing exponentially. The majority of the studies focus on the ocean which constitutes the largest ecosystem in the planet; however, viral metagenomics of freshwater, sewage and reclaimed water samples (Angly et al., 2006; Culley et al., 2006; Djikeng and Spiro, 2009; Lopez-Bueno et al., 2009; Monier et al., 2008) and stool samples (Kurokawa et al., 2007; Victoria et al., 2009; Zhang et al., 2006) have been studied. Untargeted metagenomics has been able to identify virus sequences in sewage samples. A comparison between sewage viromes from different geographic locations was previously conducted, including the United States, Nigeria, Thailand and Nepal (Ng et al., 2012). Novel full and partial genomes of viruses related to 29 eukaryotic viral families infecting vertebrates, invertebrates, and plants were characterized. Ng and co-workers suggested the use of sewage to monitor the diversity and circulation of viral pathogens within the community with focus on newly characterized viruses. Another study of sewage samples identified 21 viral families, including several human DNA/RNA viruses such as JCPyV and BKPyV, Picornaviridae, and Papillomaviridae (Aw et al., 2014). The most dominant sequences were related to bacteriophages and animal viruses were the second most abundant viral group (Aw et al., 2014). The possibility of a direct metagenomic approach for routine surveillance was also previously investigated (Furtak et al., 2016). The study showed that tomato mosaic virus was the most prominent plant virus (41.20%) and adeno-associated virus 2 was the most abundant mammalian virus (0.5%) (Furtak et al., 2016). In this study, the concentration of poliovirus from sewage increased poliovirus-specific reads in uncultivated sewage concentrate (Furtak et al., 2016). Metagenomics has been performed also to examine reclaimed water to characterize the viral community in comparison with potable water (Rosario et al., 2009). The reclaimed water contained 1000-fold more viral particles than potable water. Most of the viruses detected in the examined samples were novel viruses related to single stranded DNA and RNA viruses, and the most dominant DNA viruses were bacteriophages. The study pointed out the role of reclaimed water in the dissemination of stable viruses. The infectious risk associated with land application of sewage sludge has been investigated using shotgun viral metagenomics in five wastewater treatment plants in the United States (Bibby and Peccia, 2013a). Different types of human viruses (26 DNA and 17 RNA) were identified from nearly 330 million obtained sequences. Interestingly, the results showed a high abundance of respiratory viruses and a minor representation of enteric viruses. This was confirmed by a follow-up study that demonstrated that the majority of AdVs in sewage sludge are respiratory AdVs (Bibby and Peccia, 2013b). The study showed high degree of viral diversity in sewage samples and metagenomic results were highly reproducible. Cantalupo et al. (2011) explored viral diversity in untreated wastewater collected from United States, Spain and Ethiopia using a metagenomics approach. In addition to bacteriophages, viruses infecting human, plant, algae and insect were identified. This supports the use of untreated wastewater to study the diversity of viruses and identification of novel viruses, although we note viral diversity may be underestimated by metagenomic methods due to either database or method limitations.

While the majority of viral metagenomics studies to date have been untargeted, i.e. using shotgun metagenomic sequencing, targeted approaches may be employed to explore the diversity of a specific viral group. In these studies, a specific gene that contains phylogenetic information is PCR-amplified and sequenced. A recent study exploring HAdV diversity in Italy identified four groups in sewage (A, B, C, and F), while group F was the most dominant (Iaconelli et al., 2017). This is consistent with a prior study using a targeted approach to study adenovirus in wastewater in France found that HAdV 41 (group F) to be the most dominant (Ogorzaly et al., 2015). A targeted study in sewage sludge found that respiratory HAdV (groups B and C) were the most abundant HAdV types (Bibby and Peccia, 2013b). A targeted study in United States found that dominant EV subtypes varied between EV A and EV B seasonally, with lesser contribution from other subtypes (Brinkman et al., 2017). Finally, a targeted sequencing approach was used to explore NoV recombination in wastewater and may be capable of more rapid identification of novel NoV variants as well as identification of dominant NoV variants within the population (Lun et al., 2018). The higher resolution enabled by targeted sequencing approaches have significant potential to elucidate viral diversity that may otherwise be difficult to describe using solely shotgun sequencing.

There are many challenges with the analysis of metagenomics results due to the large amount of data produced (Spjuth et al., 2016). The ultimate sequence analysis is influenced by the read length (Prakash and Taylor, 2012). The improved read lengths offered by emerging DNA sequencing technologies, such as Nanopore sequencing, may enable complete coverage of a viral genome in a single read and enhance viral identifications from metagenomics. Nanopore sequencing has been successfully applied for viral identification in clinical samples (Greninger et al., 2015). Nanopore sequencing has yet to be widely applied in environmental samples where targets would be less enriched, potentially challenging accurate identifications. Accurate annotation of viral metagenomes is also challenged by database limitations (Bibby, 2014).

Metagenomics does not require culturing or cloning prior to sequence analysis. This means any known or unknown viruses, both culturable and unculturable, can be determined using viral metagenomic analysis. Viruses are present everywhere, and considered the most abundant biological agent on the planet. However, monitoring viral communities is a complex challenge, because far less than 1% of viral genomes have been identified so far and the majority is unknown (Bibby, 2014). Due to its sensitivity and broad range of detection, the method has great potential in viral surveillance in aquatic environment (Aarestrup and Koopmans, 2016). Notably, bioinformatic analysis of viral metagenomes is not yet standardized and depends heavily on study goals and methods, as well as location (Nooij et al., 2018; Osunmakinde et al., 2018). Due to the absence of background information of the infected hosts it is difficult to link the data obtained by metagenomics to viral genomes from the environment; therefore, identification of novel pathogens in aquatic samples is challenging (Edwards et al., 2016). In addition, it is more challenging to link metagenomic viral sequences to diseases, owing to the lack of virus isolation via cell culture (Mokili et al., 2012). Next-generation sequencing techniques have revolutionized metagenomics and the characterization of complex microbial communities gaining insight into the role of viruses in aquatic environments and increasing the number of discovered viruses. In addition, viral metagenomics has a high potential to assist in the discovery of novel viral water quality indicators, as highlighted in a recent review (Bibby et al., 2019).

7. Conclusion

- Currently, qPCR is the most commonly used method to detect human viruses in environmental samples due to its high sensitivity and specificity. However, the assay does not clearly discriminate between infectious and non-infectious viruses. Moreover, qPCR is sensitive to the inhibitors that may be co-concentrated with viruses.

- Various approaches have been developed to overcome the drawbacks of qPCR such as dye treatment, ICC-PCR, and enzymatic treatment. However, these methods are dependent on a case by case basis for the discrimination of infectious viruses. In addition, comprehensive optimization are needed still to allow multiplex detection and solve the problem of non-culturable viruses.
- Application of next generation sequencing to environmental virology has the potential to greatly increase our knowledge of viral community in terms of viral discovery and viral diversity in the environment.
- Ultimately, while existing methods have demonstrated the importance and diversity of enteric viruses in the water environment, further developments are necessary to enable more rapid and accurate methodologies for viral water quality monitoring and regulation. For example, development of on-site detection or sequencing method should be considered to provide early warning of viral contamination risk.

Competing interests

The authors declare no competing interests.

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