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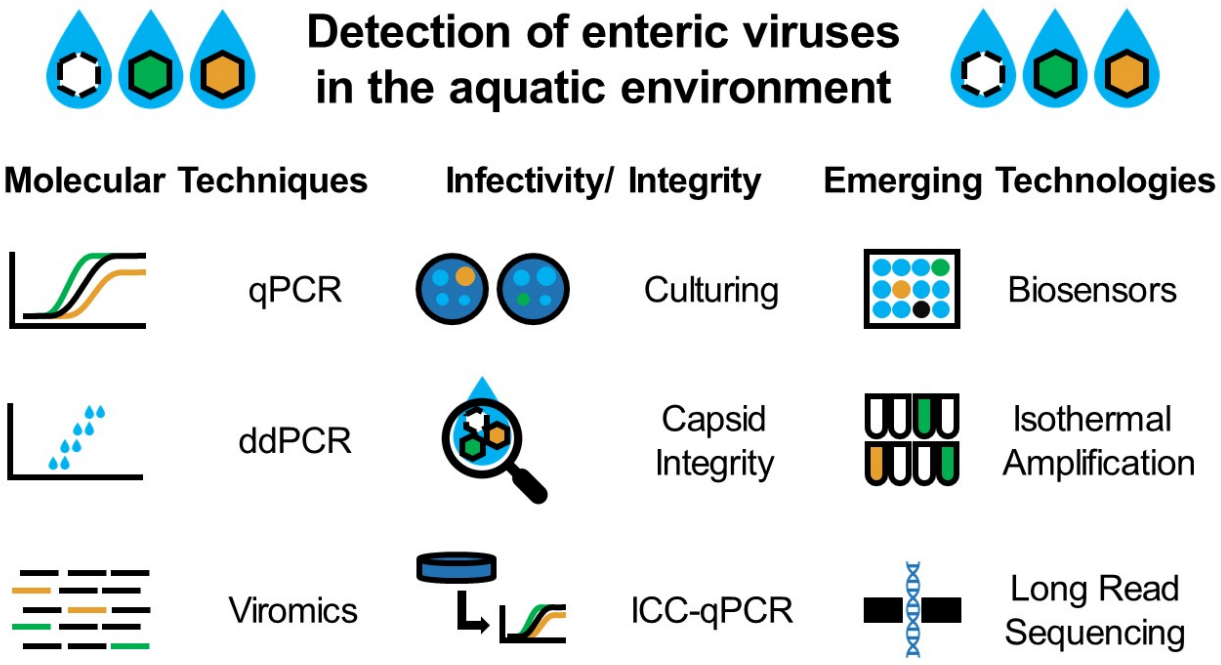
Detection of enteric viruses in the aquatic environment
Molecular Techniques
Infectivity/ Integrity
Emerging Technologies
qPCR
Culturing
Biosensors
ddPCR
Capsid Integrity
Isothermal Amplification
Viromics
ICC-qPCR
Long Read Sequencing

Emerging technologies for the rapid detection of enteric viruses in the aquatic environment

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Abstract

Due to the high diversity of enteric viruses in the environment, there is an increasing need for methods enabling the multiple detection of different pathogens. Quantitative, emerging digital PCR and isothermal amplification approaches are capable of the quantification of multiple targets, and hence are suitable for long-term monitoring and source tracking of enteric viruses in the aquatic environment. The combination of culturing with PCR-based detection enables rapid viral risk assessment, especially with host tissues capable of the propagation of several viral strains. Viability assays may provide a better understanding on viral survival than PCR-based approaches alone, however, the usefulness of these assays in wastewater and environmental water samples should be further investigated. Undoubtedly, emerging sequencing-based technologies provide invaluable data on the ecology and diversity of viruses, and, along with rapid on-site technologies, e.g. biosensors, may be implemented in viral risk assessment in the aquatic environment in the near future.

Keywords: viromics; dPCR; LAMP; viability assay; ICC-qPCR; aptasensors

1. Introduction

Enteric viruses are the major cause of gastroenteritis globally. They enter the aquatic environment via wastewater discharge, agricultural activities and landfill run-off polluting surface water, groundwater and sediment. Due to their extreme persistency, they contaminate recreational waters, drinking water sources, irrigation water and they are accumulated by shellfish. Hence, they are often responsible for water- and foodborne illnesses [1]. There are over 150 pathogenic viruses that may be found in water environments, including noroviruses, sapoviruses, hepatitis A/E viruses, rotaviruses, enteroviruses, Aichi viruses, astroviruses, adenoviruses and polyomaviruses [1]. Hence, there is a need for the simultaneous detection of multiple strains or species to better understand viral risks. The quantification process of viruses involves the concentration of environmental samples prior to detection, which results in a difficult matrix that hinders accurate detection [2]. This review focuses on recent method developments (Table 1) for the accurate detection of multiple viral targets which have been used, or may be used, for enteric viral monitoring in the aquatic environment.

2. Detection and quantification of viral nucleic acids

2.1 q(RT-)PCR and d(RT-)PCR

Polymerase chain reaction (PCR) methods targeting genes of pathogens have been widely used in environmental health research. The most common method in monitoring viruses is the real-time or quantitative PCR (qPCR) often combined with a reverse transcription step (RT-qPCR) to quantify RNA targets. The PCR reaction can be performed on a microfluidic platform, reducing the time and costs of the assay. Microfluidic qPCR have been used for the detection of multiple viral targets in water samples [3]. However, as small volumes of amplification reaction mixes and samples are used, the limit of detection can be high (e.g. 150 copies/µl) [3], which is not ideal for most environmental samples.

The emerging technology for viral quantification is the digital (RT-)PCR (d(RT-)PCR), where the PCR mix is dispersed in thousands to millions of individual wells on a chip or water-oil droplets and the target sequence quantities are calculated based on +/- signals. Quantitative/dPCR approaches are rapid and highly sensitive, enabling the strain-level detection of 1-10 genome copies (gc) within 1-4 hours. The major advantage of dPCR over qPCR is that it performs absolute quantification and hence no standards are required. Comparative studies showed that dPCR is more sensitive and less affected by inhibitors than qPCR-based approaches [4,5]. However, d(RT-)PCR has a narrower range of quantification than qPCR [5], and hence samples with high viral concentrations (e.g. wastewater samples) should be diluted prior to d(RT-)PCR for quantification.

TaqMan (probe-based) qPCR assays can be multiplexed enabling the parallel detection of 2-4 targets within one qPCR reaction well [6–8]. This can be useful for the simultaneous quantification of human and animal viruses for source tracking [9]. Multiplex qPCR assays are also available commercially and have been used to identify viral pathogens in stool samples [10]. Digital PCR assays can also be multiplexed, and a duplex assay has been used for viral detection in clinical setting [11]. The main disadvantage of the d(RT-)PCR is the high costs (either equipment – droplet dPCR or consumables – chip-based dPCR), which obstruct the wide use of the equipment in environmental research and routine monitoring.

2.3 Isothermal amplification

Isothermal amplification methods, such as nucleic acid sequence-based amplification (NASBA), recombinase polymerase amplification (RPA), helicase dependent amplification, and loop-mediated isothermal amplification (LAMP), have the potential to detect low concentration of target DNA or RNA sequences within 15-60 minutes at 37-65°C. These techniques have been used for pathogen detection in environmental samples [12], with RPA, LAMP and NASBA have been used for viral targets [13–15].

LAMP uses three primer sets which enables the creation of loop sequences and increases the number of primer binding sites with each amplification. Therefore, LAMP is highly specific and produces considerably more amplicons than PCR within a short period of time without using a thermal cycler. An RT step can easily be implemented for the detection of RNA target and it can also be multiplexed [16]. LAMP is less sensitive to inhibitors compared to PCR [13]. Due to its simplicity, specificity and reliability, LAMP could be implemented in viral water quality assessment. A microfluidic approach has also been used on water samples to detect multiple cellular pathogen targets [17], but not for viruses.

NASBA and RPA uses a combination of enzymes to rapidly amplify multiple target nucleic acid sequences. Unlike other nucleic acid amplification methods, NASBA can directly amplify from an RNA target, removing the need for an RT step. However, due to the complexity of the RPA and NASBA reactions, they may be more prone to inhibition than other techniques and can generate unreliable results [14,15,18]. More research is needed to assess the usefulness of RPA in environmental virus monitoring. A recent review suggested that NASBA has potential for further application for environmental analysis [19]. However, the difficulties in generating reliable, quantitative results and the current cost of NASBA relative to PCR and LAMP limit its adoption as a common tool for analysis of viruses in the environment [18].

2.4 High-throughput sequencing

High-throughput sequencing (HTS) can be used to survey the DNA and/or RNA of viral communities in aquatic systems without the bias of pre-selecting which viruses to detect. In this way, HTS of environmental water samples can be used to identify emergent viruses as well as known pathogens [20]. The recovery of whole genomes of uncultured viruses from metagenomics data can yield genotype-level identification and aid the design of qPCR assays for finer scale surveying [21,22]. HTS can also inform targeted amplicon sequencing studies that examine specific viral groups and yield finer resolution of their geographic distribution [23] and diversity [24].

Nonetheless, qPCR and HTS can sometimes produce conflicting evidence on the presence of specific viruses [25] and data-processing can introduce artefacts and chimeric sequences [26]. Long-read sequencing (PacBio, Oxford Nanopore) can overcome some of these limitations but it produces high error rates of up to 15% [27,28]. The major disadvantages of HTS are the costs of sequencing and the time required for bioinformatics analysis, which prohibits the use of these advanced technologies for routine monitoring.

3. Assessment of viral infectivity

The main disadvantage of all nucleic acid detection systems is the lack of information on infectivity. The culturing of human viruses requires specific equipment (e.g. CO_2 incubator, inverted microscope) and well-maintained cell lines, and is therefore rarely used in routine viral monitoring. Furthermore, *in vitro* culturing assays are not available for many human viruses and the existing methods based on the observation of cell lysis due to viral infection (i.e. cytopathic effect; CPE) may take weeks. Nonetheless, those methods have the capability to assess viral infectivity and that is crucial to understand the removal of viruses during wastewater treatment and the decay of viruses in the environment. Therefore, attempts have been made to simplify and accelerate viral culturing techniques.

Integrated cell culture (ICC) (RT-)qPCR have been used to reduce the time of culturing necessary for infectious virus detection to 1-4 days, as the increase in viral DNA/RNA levels due to viral propagation can be accurately detected by (RT-)qPCR several days before CPE is visible. Recently, these techniques have been used for the detection of enteric viruses in surface water [29,30]. The assay can be further shortened to a few hours by detecting viruses at the early stage of cell attachment [31]. The advantage of ICC-(RT-)qPCR is that one cell line can be used for the propagation of several different viral strains enabling the assessment of different targets in water samples [29,32].

The focus of research on viral culturing has been the propagation of human noroviruses. Since 2016, three methods have been developed using human B cells [33], human stem cell-derived enteroids [34] and intestinal epithelial cells [35] and zebrafish embryos [36] as hosts. All methods have been shown to result in norovirus gc increase within 2-4 days. However, these methods have rarely been used to investigate norovirus infectivity in environmental samples and their usefulness to propagate different viruses needs to be investigated.

4. Assessment of viral integrity

Due to the disadvantages of RNA/DNA and culturing-based viral detection systems, inexpensive and simple assays evaluating the integrity of the viral particles have been developed, based on the assumption that an intact virus particle is infectious.

5.1 Elimination of free viral nucleic acids

Free nucleic acids can be eliminated by enzymatic (RNase or DNase) treatment, during which the non-encapsidated viral nucleic acids are degraded, prior to PCR-based quantification. Enzymatic treatments have been shown to eliminate free nucleic acids to some extent [37]. The treatment is often coupled with proteinase K treatment, which degrades the damaged capsid proteins, enabling the nucleases to reach nucleic acids from non-infectious viral particles. However, proteinase K treatment has been shown to damage infectious viral particles as well, hence it should be used with caution [38].

Another approach for the elimination of free nucleic acids is viability treatment using intercalating dyes. These substances are able to penetrate compromised viral capsids and, when the sample is exposed to light, it covalently binds to nucleic acids preventing PCR amplification. The most frequently used dyes in environmental studies are propidium monoazide (PMA) and ethidium monoazide (EMA). Other viability treatments use substances that bind to nucleic acids without light exposure, such as platinum chloride ($PtCl_4$) and cis-dichlorodiammineplatinum (CDDP), which have

also been tested on heat/UV inactivated or chlorinated viral samples. The results suggest that these treatments eliminate the majority of free nucleic acids, however, their performance shows variations amongst different sample types and viral species [39–42]. PMA treatment can also been enhanced by the addition of surfactants [39,41,43,44] or by combination with EMA [45]. The major advantage of these assays is that they are not strain specific and hence, multiple targets can be analysed in one sample.

5.2 Capsid integrity assay

Capsid integrity assays are based on affinity binding between a protein and the viral capsid. As the capsid proteins show great variations, one assay is only suitable for a few strains or species belonging to the same family. Most research has been focusing on the capsid integrity of noroviruses, mainly genotype II, assessed using histo-blood group antigens (HBGA), including porcine gastric mucin (PGM). These proteins can be immobilised to plate wells or magnetic beads [46]. When the sample is added, viral particles bind to the proteins and the subsequent washing steps can eliminate free viral nucleic acids and inhibitors, which would affect PCR-based detection. This approach is very easy and rapid and can be applied in any laboratories, however, it may not eliminate all damaged, and hence non-infectious viruses after heat-inactivation and UV treatment [47] and seems to be less effective on norovirus genogroup I viruses than on genogroup II [48].

5. Biosensors

Biosensors transduce biological responses to measurable signals upon interaction with their target [49]. Aptamer-based biosensors (aptasensors) hold single-stranded DNA or RNA oligonucleotides (i.e. aptamers), which have the ability to bind to target DNA or proteins with high specificity and affinity, and produce a measurable signal upon binding. Aptasensors developed for norovirus detection based on electrochemical, fluorescence, colorimetric and surface plasmon resonance detection platforms [50–52] may be the most promising biosensor for viral detection in aquatic samples. Aptasensors are generally resistant to environmental inhibitors, enabling high recoveries

and low detection limits within minutes [53]. Some aptamers have the potential for the multiple detection of different norovirus strains [54]. Broadly reactive aptamers combined with biosensor technologies could provide a valuable asset for the simultaneous detection of enteric viruses in the aquatic environment.

6. Conclusion

Various methods are available for the detection of viral pathogens in the environment, however, all of them have their limitations. For rapid assessment and source tracking, PCR and isothermal amplification approaches should be used. To estimate viral persistence, culturing-based methods and virus particle integrity assays can be used, however, more comparative studies (integrity vs. infectivity) are needed for the validation of these approaches in environmental risk assessment. Amplicon and full genome sequencing, however expensive and time consuming, can detect novel and emerging viral species and strains and hence is helpful in initial risk assessment and to understand the local and global distribution of viruses for epidemiological investigations. Lab-on-chip LAMP assays and biosensors, have the capacity to detect and quantify target viruses on site within an hour. With further development, these assays could be used for environmental monitoring of common enteric virus strains, providing an invaluable tool for authorities and other stakeholders for the rapid initial water/food quality assessment and mitigation.

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196 Table 1. Summary of the commonly used and emerging methods for viral detection in environmental
197 studies. q(RT-)PCR: quantitative (reverse transcription) PCR; d(RT-)PCR: digital(reverse transcription)
198 PCR; LAMP: loop-mediated isothermal amplification; RPA: recombinase polymerase amplification;
199 HTS: high-throughput sequencing; ICC: integrated cell culture; gc: genome copies; TCID50: Median
200 Tissue Culture Infectious Dose (100-1000 gc equivalent [28]).

201

Method	**Target**	**Quantification**	**Sensitivity**	**Time to complete**
q(RT-)PCR	Up to 4 viral strains/species	Relative quantification	1-10 gc/reaction [5,7]	1-4 hours
d(RT-)PCR	Up to 2 viral strains/species	Absolute quantification	1-10 gc/reaction [5]	1-3 hours
LAMP	Up to 2 viral strains/species	Absolute quantification	2 PFU/ml [12]	15-60 min
RPA	Up to 4 viral strains/species	Non-quantitative	50 gc/reaction [15]	20 min
HTS	Non-targeted detection of any RNA or DNA viruses	Semi-quantitative*	N/A	1 week
Culturing	Non-targeted detection of viruses can be cultured in the host cell	Absolute quantification	1 TCID50/ml [31]	1-2 weeks
ICC-q(RT-)PCR	As q(RT-)PCR	As q(RT-)PCR	0.02-0.2 TCID50/ml [31]	1-4 days
Viability assay	Eliminates all free nucleic acids	Depends on detection method	Depends on detection method	1 hour + detection
Capsid integrity assay	Accumulates closely related viral strains	Depends on detection method	Depends on detection method	1 hour + detection
Aptasensors	Accumulates closely related viral strains	Relative quantification	200 virus/ml [51]	10 min

202 *HTS may enable relative abundance quantification based on the relative proportion of contigs, however, its
203 performance is highly dependent on sample preparation and bioinformatics analysis [26].

204

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Highlights

- Multiplex q/dPCR are adequate tools for long-term monitoring
- ICC-qPCR and attachment-based detection enable the rapid assessment of viral infectivity
- PMA/EMA/$PtCl_4$/CDPP may be used for the detection of potentially infectious viruses
- High throughput sequencing is an excellent tool for investigating emerging viruses
- Biosensors may be used for rapid on-site assessment and monitoring

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: