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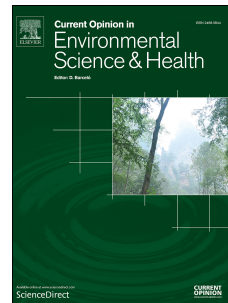
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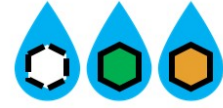
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Detection of enteric viruses in the aquatic environment



Molecular Techniques

Infectivity/ Integrity

Emerging Technologies



qPCR



Culturing



Biosensors



ddPCR



Capsid
Integrity



Isothermal
Amplification



Viromics



ICC-qPCR



Long Read
Sequencing

Journal Pre

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

3
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13 Abstract

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

25

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

27 1. Introduction

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

40 2. Detection and quantification of viral nucleic acids

41 2.1 q(RT-)PCR and d(RT-)PCR

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

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

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

67 2.3 Isothermal amplification

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

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

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

91 2.4 High-throughput sequencing

92 High-throughput sequencing (HTS) can be used to survey the DNA and/or RNA of viral communities
93 in aquatic systems without the bias of pre-selecting which viruses to detect. In this way, HTS of
94 environmental water samples can be used to identify emergent viruses as well as known pathogens
95 [20]. The recovery of whole genomes of uncultured viruses from metagenomics data can yield
96 genotype-level identification and aid the design of qPCR assays for finer scale surveying [21,22]. HTS
97 can also inform targeted amplicon sequencing studies that examine specific viral groups and yield
98 finer resolution of their geographic distribution [23] and diversity [24].

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

105 3. Assessment of viral infectivity

106 The main disadvantage of all nucleic acid detection systems is the lack of information on infectivity.
107 The culturing of human viruses requires specific equipment (e.g. CO₂ incubator, inverted
108 microscope) and well-maintained cell lines, and is therefore rarely used in routine viral monitoring.
109 Furthermore, *in vitro* culturing assays are not available for many human viruses and the existing
110 methods based on the observation of cell lysis due to viral infection (i.e. cytopathic effect; CPE) may
111 take weeks. Nonetheless, those methods have the capability to assess viral infectivity and that is
112 crucial to understand the removal of viruses during wastewater treatment and the decay of viruses
113 in the environment. Therefore, attempts have been made to simplify and accelerate viral culturing
114 techniques.

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

123 The focus of research on viral culturing has been the propagation of human noroviruses. Since 2016,
124 three methods have been developed using human B cells [33], human stem cell-derived enteroids
125 [34] and intestinal epithelial cells [35] and zebrafish embryos [36] as hosts. All methods have been
126 shown to result in norovirus gc increase within 2-4 days. However, these methods have rarely been
127 used to investigate norovirus infectivity in environmental samples and their usefulness to propagate
128 different viruses needs to be investigated.

129 4. Assessment of viral integrity

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

133 5.1 Elimination of free viral nucleic acids

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

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

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

153 5.2 Capsid integrity assay

154 Capsid integrity assays are based on affinity binding between a protein and the viral capsid. As the
155 capsid proteins show great variations, one assay is only suitable for a few strains or species
156 belonging to the same family. Most research has been focusing on the capsid integrity of
157 noroviruses, mainly genotype II, assessed using histo-blood group antigens (HBGA), including porcine
158 gastric mucin (PGM). These proteins can be immobilised to plate wells or magnetic beads [46]. When
159 the sample is added, viral particles bind to the proteins and the subsequent washing steps can
160 eliminate free viral nucleic acids and inhibitors, which would affect PCR-based detection. This
161 approach is very easy and rapid and can be applied in any laboratories, however, it may not
162 eliminate all damaged, and hence non-infectious viruses after heat-inactivation and UV treatment
163 [47] and seems to be less effective on norovirus genogroup I viruses than on genogroup II [48].

164 5. Biosensors

165 Biosensors transduce biological responses to measurable signals upon interaction with their target
166 [49]. Aptamer-based biosensors (aptasensors) hold single-stranded DNA or RNA oligonucleotides
167 (i.e. aptamers), which have the ability to bind to target DNA or proteins with high specificity and
168 affinity, and produce a measurable signal upon binding. Aptasensors developed for norovirus
169 detection based on electrochemical, fluorescence, colorimetric and surface plasmon resonance
170 detection platforms [50–52] may be the most promising biosensor for viral detection in aquatic
171 samples. Aptasensors are generally resistant to environmental inhibitors, enabling high recoveries

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

176 6. Conclusion

177 Various methods are available for the detection of viral pathogens in the environment, however, all
178 of them have their limitations. For rapid assessment and source tracking, PCR and isothermal
179 amplification approaches should be used. To estimate viral persistence, culturing-based methods
180 and virus particle integrity assays can be used, however, more comparative studies (integrity vs.
181 infectivity) are needed for the validation of these approaches in environmental risk assessment.
182 Amplicon and full genome sequencing, however expensive and time consuming, can detect novel
183 and emerging viral species and strains and hence is helpful in initial risk assessment and to
184 understand the local and global distribution of viruses for epidemiological investigations. Lab-on-
185 chip LAMP assays and biosensors, have the capacity to detect and quantify target viruses on site
186 within an hour. With further development, these assays could be used for environmental monitoring
187 of common enteric virus strains, providing an invaluable tool for authorities and other stakeholders
188 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/PtCl₄/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

Journal Pre-proof

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: