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A Review of Molecular Methods for Viral Detection in Water and Future Public Health Perspectives

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Abstract. Safe water is essential for public health, but environmental waters frequently contain viral contaminants. Molecular detection methods, particularly Polymerase Chain Reaction (PCR)-based approaches, have transformed water quality monitoring by providing rapid and sensitive analysis without the need for cultivation. This review focuses on two major technologies: quantitative PCR (qPCR) and digital PCR (dPCR). qPCR remains widely used for virus surveillance, source tracking, and infectivity assessments, while dPCR offers absolute quantification, improved tolerance to inhibitors, and greater precision for low-level targets. Both methods require quantitative criteria for performance evaluation and face some challenges, including matrix inhibition, lack of viability confirmation, and the need for standardized workflows. Future progress is expected through improved robustness, more efficient sample processing, enhanced multiplexing, and integration with risk modeling and viability assays. Continued reductions in cost and gains in throughput may broaden access to dPCR, and the complementary use of qPCR and dPCR can further strengthen water quality surveillance and public health protection.

Keywords: Viral detection, dPCR, qPCR, water environment

1. Introduction

Water plays a vital role in human life, not only for drinking but also for daily activities. Although only about 10% of global freshwater is used for household purposes, secure water access is critical for public health. In contrast, approximately 70% of freshwater is allocated for agriculture and food production, with freshwater rivers and lakes serving as key resources [1]. However, industrial development and the recent pandemic have intensified water pollution, posing severe environmental and health risks. The United States Environmental Protection Agency (USEPA) reported that around 200 million pounds of toxic chemicals are released into surface waters each year due to chemical spills [2]. The toxins can accumulate in aquatic organisms and enter the human food chain in the end, causing long-term harm [3]. Particularly concerning are heavy metals, which are highly toxic and persistent. Heavy metals can bioaccumulate in organisms and cannot degrade naturally [4]. The research shows that community or government can reduce cumulative public health impact applying countermeasure as earlier as possible [5].

Beyond chemical pollution, waterborne viruses also represent great and often underestimated threat. While it was commonly believed that viruses in water are rare and difficult to detect, meta-

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viromics has revealed their abundance and diversity [6]. Many viruses are nanometers in size and can cause infection with only a few to a thousand particles, making them highly potent [7]. Transmission can occur through ingestion, inhalation, or other routes, affecting human, animal, and plant health. The World Health Organization (WHO) has identified several waterborne viruses of moderate to high public health concern, including adenoviruses, astroviruses, hepatitis A and E, rotaviruses, noroviruses, and enteroviruses [8]. These viruses are typically associated with gastroenteritis and symptoms like diarrhea, vomiting, and fever. Monitoring viral pathogens in water environments not only helps reduce disease transmission but also enables early detection of unreported cases, providing valuable support for public health responses [9]. This review summarizes current methods for detecting waterborne viruses and highlights their significance in safeguarding public health.

2. Molecular methods for microbial detection in water

Early water microbiology relied on culture-based assays that grow colonies on selective or differential media and then count or confirm them. For example, the standard methods approach to indicator bacteria can be as simple as inoculating a 100-mL bottle and reading presence/absence or colony counts after incubation, but it is slow and skill-dependent, meaning that the operator needs to scan plates or slides, and may often misses organisms that are viable but non-culturable (VBNC) [10]. Viral testing procedures have been even more complicated. They often require filtering very large volumes of water through 1-micrometer pore size electropositive Microbial Detection System (MDS) filter cartridges, rinsing the trapped particles out with a beef-extract solution, concentrating them by acid flocculation or by using polyethylene glycol (PEG) precipitation, and then attempting to grow the viruses in mammalian cell cultures. Each of these steps can be affected by toxic substances present in the sample, and many viruses cannot grow in the available cell lines [10]. In hospital settings, culture-based methods are still important for detecting opportunistic waterborne pathogens. However, several practical problems can occur. Different types of culture media, such as tryptic soy agar (TSA) for total bacterial counts, Pseudomonas-selective agar, Burkholderia cepacia selective agar, and MacConkey agar, can produce different recovery rates. The incubation temperature also affects the results. For example, cultures grown at 30 °C may show better recovery for some species, while others grow better at 37 °C. Also, some pathogens may grow slowly, which can require up to five-day incubation before colonies appear. Even after growth is visible, further testing is often necessary to confirm the species. The matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and the use of antibiotic discs can help distinguish the bacteria with similar look, such as Stenotrophomonas maltophilia [11]. However, traditional culture-based methods have several shortcomings. The concentration of pathogens in water is often very low and the step of sample concentrating may introduce some substances that inhibit detection. Also, the sensitivity of culture methods can vary in different laboratories, which makes comparing results difficult.

2.1. Real-time quantitative polymerase chain reaction (qPCR)

Molecular detection techniques have been developed to overcome the limitations of traditional culture-based methods. Polymerase chain reaction (PCR) is used widely now to amplify specific DNA sequences and can detect the target organisms even at very low concentration [10]. When the target is RNA, such as the genome of many waterborne viruses, a reverse transcription step is carried out first. This step converts the RNA into complementary DNA (cDNA) and then uses PCR

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to amplify. This process is called reverse transcription PCR (RT-PCR) [12]. Quantitative PCR (qPCR), also called real-time PCR, incorporates fluorescent dyes or probes that monitor the accumulation of amplified DNA during each cycle, which can detect and quantify the target at the same time [13]. The cycle threshold (Ct) value, at which fluorescence exceeds a predefined threshold, is inversely related to the initial template quantity, and absolute or relative concentrations are calculated using standard curves [14,15]. Compared with the culture-based method, qPCR can shorten the time from several days to a few hours and offer higher sensitivity and specificity for genetic targets.

COVID-19 posed a severe threat to public health and highlighted the value of wastewater surveillance. According to the research by Zhao et al. [16], the authors monitored SARS-CoV-2 RNA in wastewater using qPCR with a positive-count strategy: they ran multiple technical replicates per sample, counted the fraction of positive reactions, and used a Poisson model to track community infection dynamics even when concentrations fell below the conventional limit of quantification. The wastewater signals aligned with case counts by symptom-onset date and helped anticipate epidemic waves. However, when qPCR is applied to viral monitoring, one common challenge is that it amplifies all nucleic acids present in a sample, including those from damaged viruses or free viral genomes. This can lead to overestimation of infection risks, since not all detected genomes correspond to infectious particles. To address this issue, Vu Duc et al. [17] developed a capsid integrity RT-qPCR method for surface water and tap water in Japan. The assay included a pretreatment step with sodium deoxycholate and cis-diamminedichloroplatinum (SD-CDDP), which blocks amplification of unprotected nucleic acids and ensures that only genomes enclosed in intact capsids are quantified. This adjustment provided results that more closely reflect potential infectivity. The study further demonstrated that intact pepper mild mottle virus (PMMoV) was consistently detected at higher concentrations than human enteric viruses, and that defined thresholds of intact PMMoV were predictive of the co-occurrence of infectious human viruses. What's more, instrument design can improve qPCR's accessibility. According to the paper by Sun et al. [15], a portable qPCR device with a water-cooling PCR chip and a custom fluorescence module was developed. This design shortens cycling, reduces size and cost, and enables on-site viral monitoring when conventional laboratory instruments are impractical.

Although qPCR has clear advantages, it still has several limitations. There are now many commercial platforms and kits that can simplify operation and make the process more accessible. However, they lack a single standardized workflow that applies to all sample types, and certain applications still face issues such as variability in target abundance, matrix inhibition, and operational constraints. To evaluate whether a qPCR assay produces reliable results, several quantitative criteria should be considered. First, sensitivity can be assessed by determining the limit of detection (LOD) or limit of quantification (LOQ) through serial dilutions of target DNA or RNA, ideally using certified reference materials such as National Institute of Standards and Technology Standard Reference Material (NIST SRM) 2917 [18]. Second, specificity should be confirmed by both in-silico sequence analysis and wet-lab cross-reactivity testing against non-target organisms. Third, accuracy may be evaluated by spiking samples with known target quantities or by comparing results with reference culture-based methods, ensuring deviations remain within predefined acceptance ranges. Fourth, reproducibility should be tested through replicate runs within and across batches, and, when possible, verified through inter-laboratory comparisons using consistent reference materials. Fifth, inhibition control is essential—internal amplification controls (IACs) or process controls should be included to detect and account for matrix-derived PCR inhibition. Sixth, calibration and efficiency should be checked by evaluating the linearity ($R^2 \geq 0.98$) and efficiency

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(90–110%) of the standard curve. The deviations may indicate suboptimal assay design or execution. Lastly, operational fitness, such as time-to-result, portability, and applicability in field settings, should be assessed for real-world deployment. Sivaganesan et al. [14] improved reproducibility and accuracy by incorporating certified reference materials and inactive Enterococcus faecalis process controls. Borgolte et al. [19] validated specificity through extensive laboratory and field testing, and Sun et al. [15] demonstrated how portable qPCR devices can reduce turnaround time while emphasizing the need for calibration and inter-laboratory verification before regulatory use. Overall, adhering to these performance criteria is essential to ensure the accuracy and reliability of qPCR results, and it also helps generate data that can meaningfully inform public health decisions.

2.2. Digital PCR (dPCR)

Digital PCR (dPCR) was developed to address some of the limitations of quantitative PCR (qPCR), including the need for standard curves during quantification and the lower accuracy observed when amplification inhibitors are present. In this method, a sample is divided into thousands of small reaction volumes, such as droplets, nanowells, or microchambers, so that each reaction contains zero, one, or only a few copies of the target nucleic acid. PCR amplification occurs separately in each partition. After the thermal cycling process, the instrument records the number of positive and negative partitions. Poisson statistics are then applied to determine the absolute number of target molecules in the original sample. Because the quantification is based on direct counting rather than comparison to a standard curve, dPCR can provide measurements with higher consistency between different laboratories and better repeatability over time. In addition, the partitioning process can reduce the influence of inhibitory substances that are often present in environmental water samples [13]. When the target is RNA, reverse transcription can be included before amplification to form reverse transcription digital PCR (RT-dPCR), which combines RNA-to-DNA conversion with the partitioned amplification process [20].

In wastewater virology, Roman et al. [21] applied multiplex RT-dPCR to monitor wild-type measles virus in community sewage. Their approach targeted multiple genomic regions and was able to distinguish wild-type strains from vaccine-derived signals by including a vaccine-specific assay. By applying this method to municipal wastewater, the study demonstrated that dPCR can sensitively capture low-level viral circulation, even when clinical case reports are scarce. This highlights the role of dPCR as a complementary tool to clinical surveillance, providing early evidence of ongoing transmission and strengthening measles control and elimination strategies. In another study, Tiwari et al. [13] highlighted how dPCR and RT-dPCR can enhance virus detection in water, particularly during the COVID-19 pandemic. They reported that RT-dPCR showed higher sensitivity than RT-qPCR for detecting SARS-CoV-2 RNA in wastewater samples, especially when viral concentrations were low. This advantage comes from partitioning and endpoint detection, which reduce dependence on amplification efficiency and standard curves. The study emphasized that these features make dPCR a reliable tool for wastewater surveillance, early outbreak detection and offering great benefits for public health monitoring.

Similar to qPCR, dPCR also has a standardized evaluation framework to ensure data reliability. The dMIQE (digital Minimum Information for Publication of Quantitative Digital PCR Experiments) guidelines provide specific recommendations, such as reporting the total number of accepted partitions, partition volume, and how non-detects are handled. Evaluation should also include comparison with established reference methods, validation in multiple water matrices, and reproducibility testing across operators and laboratories, as demonstrated by Sthapit et al. [20] in wastewater and drinking water studies and by Tiwari et al. [13] through the inclusion of rigorous

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positive and negative controls. Despite its strengths, dPCR faces practical limitations. Instruments and consumables are more expensive than those for qPCR, and throughput is generally lower, which can limit adoption in high-volume or resource-limited settings. Partitioning formats differ between platforms, influencing sensitivity and comparability, and dead volume can reduce detection efficiency for very low-concentration targets. Like qPCR, dPCR detects nucleic acids but cannot determine organism viability, and while more tolerant to inhibitors, it may still require sample cleanup in heavily contaminated matrices. Addressing these issues will require broader adoption of standardized workflows, inter-laboratory calibration, and the use of certified reference materials to support consistent performance across diverse applications.

3. Conclusion

This review summarized how qPCR and dPCR are being used in water-related public health, their core principles, and their future directions. qPCR is progressing toward faster and more portable instruments. One example is a portable qPCR device with a water-cooling design, which shortens cycling time, reduces instrument size and cost, and makes on-site applications more feasible. dPCR is gaining broader adoption because it provides absolute quantification and greater tolerance to inhibitors. However, the previous studies also emphasize the need for cross-platform comparability and standardized thresholds for partition calling. Field studies have shown that dPCR can be particularly effective for detecting low-intensity or rare targets, whereas qPCR remains advantageous for high-load samples and high-throughput needs [22]. In addition, dPCR can be combined with microbial source tracking markers and antibiotic-resistance assays to assess treatment performance and detect emerging risks within a single monitoring effort.

In the future, research priorities may include harmonizing workflows, establishing inter-laboratory calibration, and adopting certified reference materials to improve comparability across studies. Continued advancements in instrument design could reduce cost and increase throughput, expanding accessibility in both routine monitoring and outbreak investigations. Linking waterborne virus detection results with management decisions through risk modeling and decision-support tools will enhance the practical value of both qPCR and dPCR.

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