Review of digital PCR potential for surveillance of emerging disease from wastewater

A Dewantoro*, W C Anggundari, B Prasetya and Yopi
Centre of Research and Human Resources Development, National Standardization Agency of Indonesia (BSN), PUSPIPTEK 420, South Tangerang, 15314

*E-mail : auragadewanto@gmail.com

Abstract. Emerging infectious diseases (EID) such as COVID-19 had been widely caused massive impact for all countries in the world. The spreading of pathogens became uncontrolled and unpredictable to overcome this pandemic disease. Some non-waterborne EID also was discovered in wastewater in many countries of the world. Studies showed that digital PCR could become a powerful tool for environmental surveillance. It enables the performance of absolute quantification for nucleic acid with a high inhibitory sample, like wastewater, and potentially possibly detected a tiny quantity of pathogen residue and tracked the infectious diseases that originated from human excretions into sewage. Hopefully, with the development of this method and support of measurement and standardization, it is possible to become an effective method to overcome the digital PCR (dPCR) method challenge for surveillance of disease transmission from wastewater.

1. Introduction
Emerging infectious diseases (EID) is currently a major source of concern for human health. A newly discovered pathogen, such as COVID-19, has a high infectious rate and causes pandemics in the majority of the world's countries. Massive detections and lockdowns were carried out quickly to isolate the infected region, but we failed due to human mobility and an unprepared healthcare system. According to some studies, COVID-19 patients who received post-treatment were tested positive in their stool sample, although the throat swab specimen was negative [1], [2]. In these cases, the wastewater was contaminated by COVID-19 from human stool and urinal, but its pathogenicity is still unknown [3]–[5]. COVID-19 was found in wastewater before the first official case was confirmed in Spain [6] and in a single building in Hongkong, two days before the household was first diagnosed with quantitative PCR (qPCR) [7].

Contamination of EID by human or animal feces and urine emphasizes the importance of wastewater based epidemiology (WBE) as an environmental surveillance instrument, particularly for community health. WBE is a technique that analyzes chemical contaminants or biomarkers in wastewater samples to obtain qualitative and quantitative data on the lifestyle of people within a specified wastewater reservoir area. [8]. WBE has the potential to provide an overview of community health as a consideration of public policy. This method could also be used as a disease outbreak early warning system and a one-of-a-kind tool for discovering pandemic hotspots [8]. This approach, however, overcomes some problems associated with laboratory technical issues for high inhibitor samples such as wastewater. [9]. The most advanced of PCR technology at the moment, digital PCR (dPCR), scientifically improves the
method by improving measurement into absolute quantification when compared to its predecessor, qPCR [10], [11].

The ability to detect disease transmission through wastewater analysis using digital Polymerase Chain Reaction (dPCR) is quite promising. Other studies have shown that insect-borne EIDs such as Zika, Dengue, and West Nile virus can be detected via wastewater. [12]. This tool has a high potential for monitoring emerging diseases in wastewater samples. Based on this research, the author would like to discuss the dPCR potential for environmental surveillance, particularly for early warning of EID transmission such as COVID-19, as well as the major challenges in laboratory testing. This paper review is descriptive-analytic based on literature studies from several scientific journals and publications released by international organizations related to EID and dPCR application in the surveillance of wastewater.

2. Further investigation of EID trace contamination in sewage

The occurrence of EID has increased significantly from the late twentieth century to the early twenty-first century [13], [14]. Some researchers have reported the possibility of an EID pathogen-contaminated aquatic environment originating from infected human feces and urine (Table 1). Pathogen traces appear to persist in human sewage systems, but their pathogenicity is unknown. Pathogenicity is defined as the potential to cause disease or the quality or state of being pathogenic [15]. The Zika virus, on the other hand, has been reported to be capable of infecting the vector on a lab-scale from the diluted urine of infected hosts, which could then be transmitted back to the host. Reinfection of the Zika virus suggests that the vector could be infected again by the host excrement and spread the virus to the other host.

Respiratory EIDs such as Coronavirus (SARS CoV, MERS, COVID-19) have yet to be proven pathogenic in terms of reinfecting the host via fecal-oral or fecal-respiratory transmission or feces and urine in aquatic environments. Although, study cases in COVID-19 show active pathogenicity from feces and urine of infected patient in animal models [16], and obtainable from feces of an immunocompromised patient via cell culture [17]. Contamination of respiratory EID in wastewater raised serious concerns about whether it could infect other people with Coronavirus, but it was most likely only an RNA/DNA trace from the virus, not infectious. According to [3], the possibility of infection due to contamination of food or water from contaminated sewage is extremely low or negligible, based on the low survivability of SARS-CoV-2 in the environment. Aside from infected host excrement, untreated face masks in the respiratory EID era may pose a significant problem as a source of the contaminant in wastewater [18], [19], because infected human saliva could contain the virus and infectious in an animal model study [16].

| Disease        | Excrement | Wastewater | Pathogenicity in excrement or wastewater |
|----------------|-----------|------------|------------------------------------------|
| Zika virus     | Yes [12], [20] | Yes [21]  | Possible [12]                           |
| Dengue virus   | Yes [12]   | -          | -                                       |
| West Nile virus| Yes [22]   | -          | -                                       |
| SARS CoV       | Yes [23]   | -          | -                                       |
| MERS           | Yes [24]   | -          | -                                       |
| COVID-19       | Yes [3]    | Yes [25]   | Extremely low [3]; cell culture is possible from human feces [17] |

3. Digital PCR (dPCR) for environmental surveillance

dPCR is a sophisticated method of measuring nucleic acids for absolute quantification. Its advantage is that no references or standards are required; instead, it serves as a primary reference method for producing reference material for qPCR standard curves [26]. Sample partitioning, which divides a nucleic acid sample into many parallel individual PCR reactions [27], allows for the detection of a single
molecule and is less sensitive to inhibition (Figure 1). The complex nature of the wastewater makes it difficult to extract and quantify chemical compounds, as well as biological biomarkers. Some PCR inhibitors, such as fats, proteins, humic and fulvic acids, can interfere with PCR measurements in wastewater. Various commercial extraction kits for nucleic acid analysis will occasionally show different efficiency and consistency when extracting samples that contain a lot of PCR inhibitors, such as wastewater and sediments. The use of digital PCR will cause the partition effect of the inhibitor to be low [28]. A single molecule can be amplified exponentially in a partition reaction and assessed for each reaction to detect a gene of interest. This distinct feature, when combined with Poisson statistical data analysis, allows for greater precision than conventional PCR and qPCR, particularly when analyzing complex mixtures with high inhibitor concentrations [29].

![Figure 1. General dPCR workflow](image)

Wastewater Surveillance for EID pathogens can be performed on a variety of sewage samples. Untreated wastewater and primary sludge are two types of wastewater that can be measured. Untreated wastewater includes both domestic and non-domestic waste. The majority of primary sludge is made up of suspension particulate out of wastewater during the initial solids separation process [31]. In studies on EID measurements of municipal untreated wastewater, dPCR demonstrated greater sensitivity than qPCR, which had a lower LOQ value in combination untreated wastewater (mixed blackwater and greywater) and blackwater, but both methods were still difficult to detect the trace of nucleic acid in greywater [9]. Because of the lowered LOQ value, dPCR was able to identify more samples with low nucleic acid concentrations. Another study in aircraft untreated wastewater also suggests dPCR ability to identify more samples than qPCR [32].

The high sensitivity and precision of the dPCR approach improve the efficiency of WBE environmental analysis and monitoring. This approach will have a major impact on the management of countermeasures the spread of EID, which is difficult to predict if solely dependent on diagnostic testing. Because the pathogen's mode of transmission is unknown, EID is typically difficult to forecast in a given area however, table 1 indicates that contamination by human excrement is very likely, which can lead to wastewater contamination. Usability of dPCR can answer some of the problems of WBE by [28] such as the complexity of the matrix of wastewater, the sensitive and cost-effective method through a nucleic acid measurement approach. This advantage contributes to becoming one of the effective methods in providing more accurate results on WBE.

4. The challenges of digital PCR measurement in wastewater analysis related to emerging infectious diseases

4.1. Sampling

Many factors should be taken in sampling and analyzing wastewater for the presence of EID, such as the selection of representative locations, sample type, sample storage, and analysis method. In areas with low prevalence, quantification may necessitate larger sample volumes. It is also necessary to transport and store samples at -80 °C due to the distance between sampling and the laboratory. However, wastewater samples frozen at -80 °C had lower cycle quantification (Cq) values in multiple gene targets in qPCR, and this would also affect dPCR measurement due to the thawing process. Another technical
issue is pasteurization, would causes signal loss in wastewater samples. Pasteurization is recommended as a biosafety measure before sample processing to inactivate most pathogens in wastewater, but it is not required if the sample is processed with adequate biosafety precautions. It is recommended that frozen samples be stored in a lysis buffer to prevent PCR signal loss [33].

The number of samples used in dPCR analysis is generally less than that of qPCR. As a result, in some applications, qPCR is sometimes more sensitive than dPCR [27]. [34] also stated that the use of dPCR for waste surveillance, particularly in SARS-CoV-2 waste, showed lower sensitivity in a small number of samples. However, by combining the reactions into a single replicate, the effective input volume can be increased.

4.2. Dynamic range and cost

The maximum number of partitions also restricts the PCR's dynamic range. During the partitioning process, there may be false positives and false negatives due to non-specific PCR amplification or difficulties interpreting partitions with intermediate fluorescence levels, affecting the determination of analyte concentrations [35]. The difference in partition volume between the actual and estimated partition volume can have an impact on the calculation of absolute target molecules [36]. In some systems, the partitioning process also increases the possibility of contamination during reaction preparation. The dPCR reaction demonstrates the need for further optimization before implemented in a waste control program [34]. When compared to qPCR, dPCR instrumentation is more specialized, the workflow or testing steps are more complicated, and the cost per test is relatively higher. The absents of a reference standard maybe compensate for this problem. Another critical scenario is the partitioning of targets in chip nano wells. Gene targets with multiple copies per genome may underestimate the target during dPCR testing [37].

4.3. Dilution

Dilution is an important factor in dPCR because higher concentrations of the target molecules, result in higher variability and less precision due to loss of linearity [11], [37]. This situation is not encountered in qPCR which has better performance at this range. According to [11], dPCR best performs on the copy number of DNA standards ranging from $2.0 \times 10^0$ to $2.0 \times 10^3$ for tetrahionate reductase (ttr) gene in Salmonella, though other EID pathogen may have a different standard.

4.4. Standardization

Several studies [3], [32], [38] have demonstrated that dPCR is superior in detecting EID of various types of wastewater samples with high sensitivity values and the ability to identify the number of samples. However, [34] contradict the theory that dPCR is more sensitive and precise than qPCR especially for undiluted sample. Because of this uncertainty, reproducibility is inconsistent, making it difficult to apply for WBE. Indeed, because dPCR is still new in the context of EID surveillance on wastewater, standardized sampling and pre-analytical analysis are required to establish samples bank and database of environmental samples. The mutually agreed standard method and criteria-certified data will be an advantage for establishing a worldwide monitoring system for human pathogens [39].

5. Conclusion

In the context of EID environmental surveillance, dPCR is a cutting-edge method with capabilities comparable to qPCR. The advantage of dPCR is its sensitivity, which allows it to measure the absolute number of nucleic acid copy numbers in tiny amounts while being more resistant to inhibitors. The dPCR method can measure various types of wastewater with a higher sensitivity value without external controls. The challenges for dPCR in EID environmental surveillance include sampling, dynamic range, cost, dilution, and standardization. The use of various matrices and robust testing for each EID pathogen is also required to support the data for standardization of the dPCR method for environmental surveillance of EID.
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