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Advances in virus detection methods for wastewater-based epidemiological applications

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1. Introduction

The presence of SARS-CoV-2 in raw wastewaters has been used as a tool to determine the number of people infected in the community associated with a wastewater treatment system. This information on the SARS-CoV-2 load in the wastewater was demonstrated to be useful in predicting the occurrence and growth of COVID-19 cases [1–3]. Recent studies also showed that wastewater-based monitoring of SARS-CoV-2 complements existing COVID-19 surveillance initiatives both in locations with a low and high prevalence of the virus [4–6]. The wastewater-based surveillance has its advantages over the patient-based surveillance. These include: i) detection of pre- and asymptomatic cases, ii) community screening without required behavioural changes from the general population, iii) rapid, sensitive, and relatively low-cost mass monitoring based on a non-invasive approach, and iv) potential early warning system [5,7,8].

However, applications of wastewater-based surveillance are also limited by some factors. These include the inherent complexity of the

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wastewater matrix and the considerable dilution of SARS-CoV-2 inputs in wastewater. These are continually being addressed by studies through the development and investigation of optimum wastewater sample pretreatment steps and virus concentration methods [9–11]. The current and recent wastewater-based surveillance studies carried out worldwide have utilized reverse transcription-polymerase chain reaction (RT-PCR) and quantitative reverse transcription-polymerase chain reaction (RT-qPCR), methods that rely on the detection of nucleic acids (Fig. 1). The efficiency of these methods of detection are also affected by the presence of substances that are PCR inhibitors. Thus, pre-treatment steps, such as the virus concentration methods, to reduce the PCR inhibitors are needed prior to detection [9–11]. Recent studies detected and/or quantified SARS-COV-2 RNA in wastewater samples using real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) [12–15]. Ahmed et al. (2022b) emphasized the need to reduce false positive and false negative errors, particularly in the trace detection for viruses in wastewater samples using the PCR-based methods [8]. The latter study also emphasized the need for standardization of sampling, sample processing, and detection protocols to ensure the reliability of the PCR-based methods. The RT-qPCR and other PCR-based methods require expensive equipment (such as the thermocycler) and highly trained personnel [16]. The RT-qPCR is also time-consuming due to multiple steps such as sample processing and RNA extraction. The RT-qPCR method has been utilized for COVID-19 clinical tests, but its time and expenses requirements may limit its use for wastewater-based monitoring. For instance, Dzinamarira and co-workers have noted that the high cost of PCR in Africa has limited the number of reported SARS-CoV-2 wastewater-based surveillance [17]. Thus, alternative methods have been explored to address the limitations of PCR-based methods and improve them via advances in one or more of the following aspects: rapidity, simplicity, cost, sensitivity, and specificity.

This paper aims to review the recent developments in PCR-based methods and non-PCR-based methods to detect the SARS-CoV-2 RNA in wastewater samples. It also aims to discuss and compare newly proposed alternative methods of detection of SARS-CoV-2 in wastewater including nucleic-acid and non-nucleic-acid based detections (Table 1). The paper highlights the recent developments that have aimed to provide rapid, sensitive, specific, low-cost assays for the detection and/or quantification of SARS-CoV-2 in wastewater, even in resource-limited settings. In addition, this paper also presents emerging methods such as the capsid integrity based assays—the application of which using wastewater samples is still limited. In addition, as of the time of writing, there is no published review yet of the alternative methods applied/proposed for the detection/quantification of SARS-CoV-2 variants, specifically in wastewater samples. The paper aims to review this emerging aspect of monitoring SARS-CoV-2 dynamics in wastewater.

2. Improvements in PCR-based methods

RT-qPCR assays have been widely applied for the detection of SARS-CoV-2 RNA in wastewaters (Fig. 2). The limitations of this type of assay have led to the development of alternative PCR-based assays such as digital polymerase reaction (dPCR) assays. This section discusses the current applications of dPCR for SARS-CoV-2 monitoring in wastewater and compares their performance with that of RT-qPCR, advantages, and limitations in actual applications. A modification of the RT-qPCR assay that allows obtaining information about the viability of SARS-CoV-2 is also discussed.

2.1. Digital polymerase reaction (dPCR)

An improvement on the PCR-based methods is based on the technology of digital polymerase chain reaction (dPCR, Fig. 2c). The main advantages of the RT-dPCR over the RT-qPCR are its non-reliance on the calibration curve for direct absolute quantification, and reduced effects of PCR inhibitors [29]. Greater analytical sensitivity was observed in the RT-dPCR for the detection of SARS-COV-2 in clinical samples [30] and in

![Fig. 1. Depiction of RT-PCR process (a) and molecular pathway of PCR (b).](image-url)
In the study by Ref. [18], the obtained LODs for the CDC N1 and N2 assays in the RT-dPCR platform were 2.9 and 4.6 genome copies/reaction, respectively, while in the RT-qPCR, higher LODs were obtained (CDC N1 assay: 14 genome copies/reaction, and CDC N2 assay: 10 genome copies/reaction). Another study, which examined the quantification of SARS-CoV-2 RNA in untreated wastewater influent, showed that the reverse transcription droplet digital polymerase chain reaction (RT-ddPCR) approach had a lower LOD of 0.066 copies/μL template compared to the value of 12.00 copies/μL observed in the RT-qPCR [19]. Flood et al. (2021) reported similar results, in which the higher sensitivity of RT-ddPCR was attributed to the greater PCR efficiency even at lower target gene concentrations and to the reduced influence of the PCR inhibitors. This is important in the wastewater-based surveillance of SARS-CoV-2 since viral loads may be low in wastewater due to dilution and the wastewater matrix has high levels of PCR inhibitors. Another important finding from

### Table 1
Methods of detection of SARS-CoV-2 in wastewater and potential alternative methods.

| Method                          | Sample                | LOD                        | Concentration Method                                           | Volume of Template/Reaction mixture | References |
|---------------------------------|-----------------------|----------------------------|----------------------------------------------------------------|-------------------------------------|------------|
| **Nucleic acid Detection (PCR-based)** |                       |                            |                                                                |                                     |            |
| RT-qPCR                         | Domestic Wastewater   | 10–14 genome copies/reaction | 50 mL sample concentrated using rapid concentrator (Concentrating Pipette Select™) | 20 μL reaction mixture               | [18,19]    |
| RT-dPCR                         | Domestic Wastewater   | 2.9–4.6 genome copies/reaction | 50 mL sample concentrated using rapid concentrator (Concentrating Pipette Select™) | 40 μL reaction mixture               | [18]       |
| RT-ddPCR                        | Domestic Wastewater   | 0.066 copies/μL template   | Adjustment of pH to 3.5 and inoculation with MgCl₂·6H₂O         | 5 μL template, 25 μL reaction mixture | [19]       |
| Electrochemical Biosensor with PCR | Domestic Wastewater | 0.4 pg/μL                  | Centrifugal ultrafiltration                                     | 25 μL reaction mixture               | [20]       |
| **Nucleic acid Detection (non-PCR-based)** |                       |                            |                                                                |                                     |            |
| RT-LAMP                         | Domestic Wastewater   | 0.4 copies/μL              | Centrifugal ultrafiltration                                     | 25 μL reaction mixture               | [21]       |
| Surface water,                  | Domestic Wastewater   | 0.0093–9.3 copies/μL       | Removal of large impurities only by passing 1–100 mL sample through 80 nm filter | 30 μL reaction mixture               | [22]       |
| RT-LAMP/CRISPR-Cas12            | Nasopharyngeal Swabs  | 5–10 copies/reaction       | Direct RNA extraction was conducted                            | 100 μL reaction mixture              | [23]       |
| Multi-branch rolling circle amplification (mBRCA) with time-resolved emission spectroscopy (TRES) | Domestic Wastewater | 75–88 copies/μL            | Centrifugation                                                  | 100 μL reaction mixture              | [24]       |
| **Non-nucleic acid Detection**  |                       |                            |                                                                |                                     |            |
| Mass-spectrometry (protein detection) |                       | Not determined              |                                                                |                                     | [25]       |
| Biosensors (binding of specific antibodies with SARS-CoV-2 protein) | Domestic Wastewater | 2.42 × 10³ copies/mL | No concentration method                                       | Not reported                         | [26]       |
| Nasopharyngeal Swabs/           | Culture Medium        | 1 fg/mL                    | No concentration method                                       | 20 μL sample                         | [26]       |
| Nanosensor (binding of lanthanide-doped carbon nanoparticles with SARS-CoV-2 protein) | Domestic wastewater | 1.52 copies/μL             | No concentration method                                       | 100 μL sample                         | [27]       |

RT-qPCR: reverse transcription polymerase chain reaction; RT-dPCR: reverse transcription digital polymerase chain reaction; RT-ddPCR: reverse transcription droplet digital polymerase chain reaction; RT-LAMP: reverse transcription loop-mediated isothermal amplification; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats.

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![Fig. 2](image-url) Depiction of traditional polymerase chain reaction (PCR), quantitative or real time PCR (qPCR) and digital PCR (dPCR). Reproduced with permission (Creative Commons Attribution (CC BY) license) from Quan et al. (2018) [28].
the latter study was that RT-ddPCR generally produced lower coefficient of variations compared to those observed in RT-qPCR, regardless of the combinations of concentration methods and sample types [9]. Most studies have reported reduced effects of PCR inhibitors using RT-ddPCR. However, Graham et al., have also observed the influence of PCR inhibitors in an RT-ddPCR assay used to detect SARS-CoV-2 RNA in solids settled from urban wastewater [31]. This was attributed to the unique characteristic of the wastewater sample, notably its treatment with FeCl₃, a PCR inhibitor. The latter study demonstrated that testing wastewater samples for PCR inhibitors and applying sample processing methods to reduce the effect of PCR inhibitors is still necessary even for RT-ddPCR.

An important application of RT-ddPCR is that it may be used to identify mutations in SARS-CoV-2 variants. The RT-ddPCR method divides the sample and reagents into approximately 20,000 droplets and each droplet/partition acts as an individual PCR microreactor. The partitioning allows the concentration of target genes in the micro-reactors [28,32]. This makes the RT-ddPCR capable of detecting rare mutations that are specific to variants of concern [28,32]. Heijnen et al. (2021) demonstrated that RT-ddPCR may be utilized to accurately detect and quantify mutations in SARS-CoV-2 variants of concern in raw sewage samples from the cities of Amsterdam and Utrecht in The Netherlands [32].

2.2. Capsid integrity-based RT-qPCR assays

A limitation of the RT-qPCR method is that it only detects the presence of the SARS-CoV-2 genome and does not provide information about the viability of the virus. With the goal of inferring the viability of the SARS-CoV-2 virus in wastewater samples via RT-qPCR, pretreatment with capsid integrity reagents such as dyes, enzymes, or metal compounds have been explored (Fig. 3) [33–35]. Pretreatment with propidium monoazide (PMA), a dye that binds with viral RNA when the viral envelope is damaged, was used by Wurtzer et al. (2021) for the SARS-CoV-2 integrity assay [35]. In the study of Ganh et al., the pre-treatment with three capsid integrity reagents (ethidium monoazide (EMA), PMA, and cis-dichlorodiammineplatinum (CDDP)) was proposed to discriminate intact viruses from those with damaged capsid [33]. Monteiro et al. (2022) developed a viability RT-qPCR for the selective detection of intact SARS-CoV-2 in secondary-treated wastewater by pre-treatment with enzymatic (nuclease) and viability dye (Reagent D) [35]. The capsid integrity reagents bind with the viruses with damaged envelopes, resulting in their non-detection by RT-qPCR. The latter studies have shown the potential of capsid integrity-based RT-qPCR assays to estimate the infectious risk associated with SARS-CoV-2 contaminated wastewaters. A limitation of this type of assay is that viruses that have been inactivated by UV treatment would still be detected by the capsid-integrity based RT-qPCR assay [34]. Inactivated viruses may still have their capsids intact resulting in non-entry of capsid-integrity reagents, and subsequently in potential overestimation of the actual number of infectious viruses. There is a need to examine the effect of different factors, such as pH, wastewater temperature, and mixing conditions, among others, to the efficiency of the assays, further studies on the application of this type of assay in wastewater samples are needed [34,35].

2.3. Nucleic acid biosensor coupled with PCR for SARS-CoV-2 detection

An electrochemical biosensor that is capable of detecting SARS-CoV-2 N gene amplicons was developed by Kumar et al. (2021) for use in wastewater-based monitoring of SARS-CoV-2. The sensor functions based on the intercalation of methylene blue (MB) with DNA, and detects an increase in the electrochemical voltammetric current that results from the adsorption of the MB-DNA complex on a printed circuit board (PCB) electrode [20]. The sensor was reported to be able to detect SARS-CoV-2 gene amplicons at concentrations as low as 10 pg/µL. In addition, the detection system presented by Kumar et al. (2021) utilized a portable mini-PCR instrument, which did not require the qPCR reagents, before placing the amplicons on the PCB electrode. The low-cost thermocycler, together with the low-cost PCB electrode, were suggested to be advantageous for wastewater-based surveillance of SARS-CoV-2 in low-resource settings.

3. Non-PCR-based methods: applications and recent developments

Alternative methods that do not require thermal cycling for amplification (for PCR) have been developed for use in the detection of SARS-CoV-2 in environmental matrices, including wastewater. These include assays that rely on the detection of nucleic acid and non-nucleic acid biomarkers such as SARS-CoV-2 protein.

3.1. Nucleic acid-based methods

3.1.1. Non-PCR-based methods: applications and recent developments

The Reverse Transcription-Loop-mediated Isothermal Amplification

Fig. 3. Workflow of capsid integrity-based RT-qPCR assays. Reproduced with permission (Creative Commons CC-BY license) from Leifels et al. (2021) [36].
The method, and colourimetric RT-LAMP method was specific to the N-gene target [37]. et al. (2022) reported a 95% LOD that is 23 times higher than that obtained in wastewater samples [21,22,38,39]. Amoah et al. (2021) reported the ALOD of an assay which relies on colour change interpretation. This may be a limitation since some studies observed largely indistinguishable colour changes, and false negatives when using some clinical samples at low viral loads [37]. To date, the indistinguishable colour changes have not yet been examined in the use of RT-LAMP for SARS-CoV-2 detection in wastewater samples. Several recent studies reported qualitative detection of SARS-CoV-2 in wastewater samples using the colourimetric method [38,39]. However, the limitation of the colourimetric measurements (especially in turbid wastewater samples) was shown to be overcome using the fluorescence-based RT-LAMP method. A recent study demonstrated the absolute quantification of SARS-CoV-2 in surface water and wastewater samples using a fluorescence-based RT-LAMP method [22]. In the latter study, absolute quantification was implemented by the imaging and quantifying of the fluorescence signals. In surface water samples, the lowest detectable concentration of SARS-CoV-2 was 0.0093 copies/μL using the RT-LAMP-based method. The fluorescence-based RT-LAMP method was demonstrated to be potentially useful for field testing since it was integrated with a smartphone imaging technology and with machine learning-based image processing for the quantification [22]. The effect of amplification inhibitors in wastewater matrices on the RT-LAMP assay has not yet been studied in sufficient detail. Only one study has so far demonstrated the effect of inhibitors to a RT-LAMP-based assay, in which the presence of small particles in the influent and effluent wastewater samples contributed to higher concentrations of inhibitors and resulted in the value of the lowest detectable concentration of SARS-CoV-2 in wastewater at 9.3 copies/μL, which was higher compared to the value obtained in samples with less number of particles [22]. However, removal of solids before the analysis may lead to loss of material, lower viral count, or a false negative result. There is a need to further study the sample pre-treatment steps needed to reduce the effect of inhibitors while ensuring minimal loss before the RT-LAMP assay.

3.1.2. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based assays

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based detection of SARS-CoV-2 relies on the detection of the nucleic acid. The method involves RNA extraction and amplification, binding of CRISPR/Cas complex to the target region, and the subsequent collateral cleavage of the surrounding fluorescence reporter nucleic acids (Fig. 4) [40]. The method is coupled with isothermal amplification such as RT-LAMP which eliminates the need for PCR equipment. Ali et al. (2020) developed an assay which integrated RT-LAMP and CRISPR-Cas12 for the detection of SARS-CoV-2 from nasopharyngeal swab samples. In the latter study, a 1-h detection time using the combined RT-LAMP and CRISPR-Cas12 assay was reported, which is shorter than that in the case of the average RT-qPCR workflow. The sensitivity was also determined to be comparable to that obtained in the RT-qPCR (RT-LAMP/CRISPR-Cas12 LOD: 10 RNA copies/reaction, CDC RT-qPCR LOD: 5 copies/reaction). The assay was specific to SARS-CoV-2 since the Cas12 cuts DNA only in the presence of the SARS-CoV-2 sequence. Although the RT-LAMP/CRISPR-Cas12 assay was developed for clinical samples, it may have a potential for use in the detection of SARS-CoV-2 in wastewater samples. The portable and low-cost detection system in the latter study has the potential for in-field deployment not only as a point-of-care diagnostic tool but also as an on-site method for SARS-CoV-2 detection in wastewater.

3.1.3. Rolling circle amplification (RCA) assays

The study of Chen et al. (2022) presented the use of a nucleic acid
SARS-CoV-2 sequences (RdRp, ORF1ab and N) in spiked domestic amplification (RCA) technology has been used in the development of a detection method which does not require reverse transcription to M.V.A. Corpuz et al.

The observed LODs of the method used in the latter study for SARS-CoV-2 detection in sewage samples were in a range of 75 copies/μL to 88 copies/μL, which values are higher than the reported LODs of RT-LAMP based assays. However, aside from the elimination of reverse transcription, another advantage of the method integrating RCA with TRES is the elimination of short-lived fluorescence background noise from the sample [44]. Only one study has so far used RCA for the detection of SARS-CoV-2 in sewage samples and further studies are needed to improve the method sensitivity and to test the method on non-spiked sewage samples. The integration of RCA assays with paper-based and electrochemical sensors has also been previously reported for rapid detection of various targets in environmental samples [43]. This integration with the paper-based and electrochemical sensors may be extended to the detection of SARS-CoV-2 RNA in wastewater samples in future studies.

### 3.2. SARS-CoV-2 protein-based detection

**Detection of SARS-CoV-2 protein**

Several studies have developed mass spectrometry (MS) assays to detect SARS-CoV-2 proteins from clinical samples such as gargle solutions, and nasal swabs [47,48]. While mass spectrometry is suitable in principle for the analysis of viral proteins [49], no established suitable mass spectrometry-based assays for use in the detection of SARS-CoV-2 in wastewater samples have been developed so far. There is only a limited number of studies that has reported the use of an MS-based proteomics approach to detect SARS-CoV-2 structural and non-structural proteins in influent wastewater samples [25,50]. In the latter study, the non-structural protein pp1ab was found to be a potential alternative biomarker in sewage samples for monitoring of SARS-CoV-2. The advantages of the MS method including its potential for field deployment (due to possible miniaturization of MS equipment) may be useful for SARS-CoV-2 monitoring in wastewater [51].

An integrated cell culture-mass spectrometry (ICC-MS), in which strain-specific viral peptides of reovirus were identified, has been applied by Ye et al. (2019) to detect proteins of infectious viruses in wastewater [52]. The use of mass spectrometry eliminates the step of primer design, which is characteristic of PCR-based methods. Mass-spectrometry could be potentially used for the detection of SARS-CoV-2 proteins in wastewater. Measurements of the SARS-CoV-2 structural proteins (Nucleocapsid (N), S, Membrane (M), and Envelope (E) proteins) in several wastewater samples using mass-spectrometry can deliver more information on the relationship between viral load and infectivity [53]. Aside from sensitive and specific detection of SARS-CoV-2, high-resolution MS was shown to be capable of detecting SARS-CoV-2 VOCs [49], as discussed in more detail in Section 4.

MS methods have not yet been consistently applied for WBE, and more efforts need to be made to develop MS methods for specific detection of SARS-CoV-2 proteins and peptides in the wastewater matrix [54].

#### 3.2.2. Biosensors for SARS-CoV-2 protein detection

Several biosensors detecting SARS-CoV-2 proteins in clinical samples have been recently developed. Mavrikou et al. (2020) and Seo et al. (2020) developed biosensors that function based on the binding of SARS-CoV-2 S protein to a specific antibody [55]. However, these sensors were developed for clinical samples only and required the use of a specific antibody [45]. Some studies have aimed to develop biosensors that do not require a specific antibody. One approach that was presented was the use of a nanosensor, in which the SARS-CoV-2 N and S proteins were recognized by 3D nanosensor surfaces [56]. Though the nanosensor in the latter study was tested in a biofluid (saliva) matrix, its applicability for wastewater analysis needs to be explored further. A recent study developed a fluorescence sensing array using lanthanide-doped carbon nanoparticles (LnCNPs) for the detection of SARS-CoV-2 protein in wastewater samples [27]. The method was based on the selective binding of the LnCNPs with the spike (S) glycoprotein of SARS-CoV-2. The relative fluorescence emission intensities of the LnCNPs (lanthanides: Gd(III), Pr(III), and Yb(III)) before and after the addition of viral sample were used to characterize the fluorescence signature of the LnCNPs against SARS-CoV-2. This recent study by Alafeef et al. (2022) showed that the newly developed sensor coupled with a pattern recognition algorithm exhibited the following advantages: i) high sensitivity (LOD of 1.52 copies/μL), ii) selectivity in the detection of SARS-CoV-2 over other species (H1N1 virus, SARS-CoV-1, E.coli, B.subtilis, and S.mutans) due to differences in fluorescence response patterns, and iii) elimination of the need for an antibody [27].

### 4. Methods of detection of SARS-CoV-2 variants of concern (VOCs) in wastewater

Detection of SARS-CoV-2 variants of concern (VOC) in wastewater has emerged as an important aspect of COVID-19 wastewater-based surveillance (see Fig. 5). Whole-genome sequencing has been used to track the circulation of variants in wastewater globally [57–61]. Crits-Christoph et al. (2021) demonstrated that viral genome sequencing of SARS-CoV-2 (using high-throughput sequencing) from municipal wastewater may provide information on SARS-CoV-2 variants circulating within a selected region and may give evidence of the recent introduction of viral strains from outside a region before they are detected through local patient-based sequencing [59]. Mutations specific to the B.1.1.529 (Omicron) were detected in wastewater of four states in the United States of America, which indicated that the VOC was widely prevailing in the areas more than what was indicated in patient-based testing [62]. Sequencing also confirmed the presence of the B.1.1.529 (Omicron) variant in the wastewater of a commercial passenger aircraft and agreed with the result of the analysis of nasopharyngeal swabs from passengers [63]. However, whole-genome sequencing is also characterized as expensive, time-consuming, and requires special equipment and technical skills [64].

Alternative approaches have been developed to monitor SARS-CoV-2 VOCs in wastewaters. VOCs have characteristic mutations at the S glycoprotein, which affects viral infectivity [64,66]. Thus, methods were developed to detect mutations specific to the VOCs as an indicator of their presence in wastewaters. These methods include: i) allele-specific RT-qPCR (AS RT-qPCR), ii) RT-ddPCR, iii) nested RT-PCR assay coupled with Swinger sequencing, and iv) mass-spectrometry.

Lee et al. (2021) developed an open-source method based on an AS RT-qPCR to detect and quantify the SARS-CoV-2 B.1.1.7 (Alpha) variant from wastewater samples [67]. The presented assay targeted S protein mutations that are characteristic of B.1.1.7 (HV69/70del, Y144del, and A570D). The latter study also showed that the B.1.17 assays produced
negligible false positive rates (only 1 out of 30 replicates), exhibited low cross-reactivity, and was not affected by significant quantities of wild-type (WT) background RNA. As discussed in Section 2.1, the partitioning in the RT-ddPCR makes it suitable for the detection of mutations specific to VOCs as demonstrated by Heijnen et al. (2021) [32]. G. La Rosa et al. (2021) presented a nested RT-PCR assay targeting mutations in the SARS-CoV-2 S protein-coupled with Sanger sequencing as a rapid screening method for the presence of VOCs present in urban wastewaters [64]. The short nested RT-PCR assays were able to detect key nucleotide changes in 20I/501Y.V1 (United Kingdom) and 20 J/501Y.V3 (Brazil) in urban wastewaters in Umbria and Abruzzo (Italy), which were regions where outbreaks were occurring during the sampling period. The latter study noted that this approach may serve as a rapid screening method to identify which should be submitted for whole-genome sequencing and to provide information for decision-making related to enhanced clinical monitoring [64]. A limitation of the approaches presented in the latter studies is that the methods are targeting mutations of specific variants and will not be able to discover new variants in wastewater. However, the assays may be extended to the tracking of mutations of variants that have been pre-determined through patient-based surveillance. D’Agostino et al. (2022) proposed a PCR-based allelic-discrimination assay panel to detect SARS-CoV-2 genotypes in samples including wastewater [68]. The study demonstrated that the assay could detect mutations in up to 10 viral genome positions at once. A preamplification step was added, in which a PCR reaction containing all genotype assays in one mix was conducted, which allowed detection of variants at low levels (qPCR Ct values up to 38.5) in wastewater samples. AS RT-qPCR has also been shown by a study to be capable of detecting multiple (8) SARS-CoV-2 variants in wastewater samples based on 12 mutation sites of the S protein [69]. The latter study showed that the applicability of AS RT-PCR may be extended to the detection of new variants in the future by the adjustment in the combination of signature mutation sites.

A recent study has integrated nested PCR and liquid chromatography-mass spectrometry (LC-MS) in the detection of the SARS-CoV-2 Alpha, Delta, Omicron variants in wastewater samples [70]. The MS method was used to detect the VOCs based on the differences of their molecular weights and also eliminated the need to develop allele-specific primers or probes. In the latter study, the nPCR-LC-MS method was shown to exhibit higher selectivity in the detection of VOCs compared to the AS RT-qPCR. However, this method still relies on PCR as in those used in the previously discussed studies.

A non-PCR-based MS-based method may be a good alternative for the detection of SARS-COV-2 variants. Mann et al. (2021) demonstrated the use of mass mapping using high-resolution MS that was employed to detect and distinguish SARS-CoV-2 VOCs [49]. The principle relied on peptide signatures that had unique characteristic masses. These signatures can be used to identify and distinguish the variants, eliminating the need for gene or protein sequencing. This approach has the potential for use in the mapping of SARS-COV-2 VOCs in wastewater. However, this method also requires that further studies to standardize protocols for initial isolation of the S protein [49]. This method has been applied to clinical samples and future studies are needed to examine its application in wastewater samples for the detection of SARS-CoV-2 variants.

5. Challenges and future perspectives

Wastewater-based surveillance of SARS-CoV-2 has been applied in several countries, in which the viral load in wastewater has been compared with the number of clinical cases (see Fig. 6). While there is little doubt that this approach can be highly useful worldwide, only a limited number of studies were conducted in low-income countries. The main limitations for a more widespread and consistent use of WBE include the costs associated with the conduct of PCR-based tests. This highlights the need to develop alternative methods and to aim to continue studies on such methods until full applications in SARS-CoV-2 wastewater-based monitoring are enabled in both developed and low-income countries. A limited number of studies have presented biosensors for SARS-CoV-2 detection in the wastewater matrix [20,27]. These biosensors proposed for use in wastewater-based surveillance of SARS-CoV-2 are still in the proof-of-concept stage and still need further development and optimization. Full application of these biosensors for monitoring of SARS-CoV-2 presence in wastewaters generated in communities characterized by a wide range of populations will be advantageous even in low-resource settings. Nourinejad et al. (2021) proposed the use of a system that will use sensors in sewage manholes for accurate, fast, and inexpensive detection of the prevalence of SARS-CoV-2 in communities [71]. The same group of researchers proposed an
algorithm that helps determine the locations of manholes to support faster identification of neighbourhoods that need enhanced patient-based testing [72]. However, the sensors for use in this system are yet to be developed and tested in field conditions. This application is also limited to areas with structured sewage networks and may not be applicable to those locations in developing countries, where households may not be connected to sewage networks.

A few studies have presented the potential for the detection of SARS-CoV-2 variants in wastewater samples used to map the circulation of VOCs in the exposed communities and to provide information about the entry of new SARS-CoV-2 variants to specific regions. This can also provide data to identify the areas that need more intensive clinical surveillance due to the presence of VOCs. Further development of more rapid, sensitive, specific, and low-cost alternative methods to detect SARS-CoV-2 VOCs in wastewaters is still needed to complement the whole-genome sequencing conducted on clinical samples.

6. Conclusions

This review paper analyses improvements in the PCR-based methods and developments in alternative assays suitable for the detection of SARS-CoV-2 in wastewater. Wastewater based-surveillance may provide an efficient, sensitive, and cheaper mass monitoring of the prevalence of COVID-19 in a target population.

The development of alternative rapid, sensitive, specific, and low-cost detection methods, such as the use of biosensors to detect SARS-CoV-2 proteins will increase the potential of monitoring SARS-CoV-2 in wastewater as an epidemiological tool even in resource-limited settings. However, most of these biosensors are still applied to clinical samples. There is still a need to optimize this type of method until it can be fully implemented using wastewater samples.

Further progress is needed in the development of methods for estimating the viability of SARS-CoV-2. Capsid integrity-based methods need to be further examined in wastewater samples. Studies on the assessment of the effects of different factors/wastewater conditions to the efficiency of this type of assay are also needed.

Alternative methods for detection of SARS-CoV-2 VOCs in wastewater have also emerged (PCR-based methods and mass-spectrometry-based). However, further studies are needed particularly on the extension of the application of the methods to detection in wastewater of new variants in the future. In addition, there is also a need to further assess the sensitivity/selectivity of the different methods in the detection of different VOCs in wastewater.

Declaration of competing interest

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.

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