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Analytical methodologies for the detection of SARS-CoV-2 in wastewater: Protocols and future perspectives

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A B S T R A C T

In March 2020 the World Health Organization announced a pandemic outbreak. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative pathogen for the coronavirus disease-19 (COVID-19) pandemic. The authorities worldwide use clinical science to identify infected people, but this approach is not able to track all symptomatic and asymptomatic cases due to limited sampling capacity of the testing laboratories. This drawback is eliminated by the Wastewater-Based Epidemiology (WBE) approach. In this review, we summarized the peer-reviewed published literature (available as of September 28, 2020), in the field of WBE. The commonly used steps (sampling, storage, concentration, isolation, detection) of the analytical protocols were identified. The potential limitations of each stage of the protocols and good practices were discussed. Finally, new methods for the efficient detection of SARS-CoV-2 were proposed.

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1. Introduction

In December 2019, the Chinese health authorities reported a number of severe human pneumonia cases of unknown origin, linked to seafood wholesale market in Wuhan, Hubei Province, China. The novel beta coronavirus is the causative pathogen of the coronavirus disease-2019 (COVID-19) [1], named SARS-CoV-2 [2]. It has been detected in the vast majority of countries worldwide, resulting to the announcement of COVID-19 outbreak a pandemic by World Health Organization (WHO) in March 12, 2020 [3].

Considering the lack of vaccines and effective treatment against SARS-CoV-2, accurate and massive diagnostic tests to identify and isolate infected individuals, as well as in depth epidemiological analysis seems to be the only way for an evidence-based approach. Several research groups have developed numerous clinical assays for the detection of SARS-CoV-2 [4,5] using various analytical techniques, such as RT-qPCR, ddPCR, nested PCR, LAMP and CRISPR assays. All these techniques are characterized of high sensitivity and specificity and could be applied as complementary tools in many scientific disciplines, such as environmental sciences and forensics. An emerging field of application of these techniques could be the wastewater-based epidemiology (WBE).

The development of appropriate analytical protocols for the detection of SARS-CoV-2 in wastewater could be of high importance, since measurements can help to establish an early-warning

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system that would be able to monitor the spread and severity of the infection at a community level [6,7]. WBE is accompanied with several advantages compared to the traditional public health approaches, such as unbiased results including asymptomatic cases, real time epidemiological information about public health and lifestyle and protection of the anonymity of individuals [8]. Furthermore, one of the most significant benefits is its cost effectiveness, since the sampling is representative of a catchment community allowing wide surveillance that could identify critical changes of the infection status without selection bias [9]. WBE has proved to be an essential tool for the consumption of drugs of abuse [10,11], pharmaceuticals [12], public health related substances [13–15] and the exposure to industrial chemicals [16] and pesticides [17]. Recently, many researchers tried to expand the field of WBE towards to community-wide infectious diseases with the detection of genetic material (DNA, RNA) or biochemical markers linked to physiological responses (proteins) [18].

In this critical review, all steps (sampling, concentration step, extraction and detection) of the analytical methods for the detection of SARS-CoV-2 in wastewater are discussed. Moreover, analytical methods used in clinical science are proposed to the field of WBE.

2. Wastewater-based epidemiology (WBE)

The first publication reporting the detection of SARS-CoV-2 was performed in Australia [19] and until today many researchers have successfully confirmed the presence of SARS-CoV-2 in wastewater worldwide (i.e. [20–24]). The used protocols consisted mainly from the following four steps: sampling, storage, concentration step, extraction and detection.

2.1. Sampling

One of the most critical steps that drastically affects the results of the analysis is sampling. According to the “Best Practice Protocol” that was validated by the SCORE COST Action ES 1307, the wastewater intended for chemical analysis should be sampled as a 24 h composite sample [25,26]. Excretion profiles may vary significantly throughout the day/days because of the short half-lives of the contaminants of emerging concern (CEC). Indeed, the half-life of SARS-CoV-2 at ambient conditions (20°C) can be estimated to range between approximately 4.8 and 7.2 h [27]. The same sampling strategy is also suggested for the analysis of SARS-CoV-2 to assure the representativeness and reduce uncertainty of the back-calculation. Although, this was the ideal procedure to perform sampling for WBE, according to the reviewed scientific literature (Table 1) most of the research groups performed grab sampling, which is of high concern. Parameters of the WWTPs such as flow rate, total volume of wastewater, temperature etc. are not considered by grab sampling. Therefore, the representativeness of the results is not assured by grab sampling. This limitation could be overcome by the use of 24 h flow/time proportional composite samples. Another critical factor is the sample volume that is used by the developed sample protocols (Table 1). Low sample volume in combination with low recovery could lead to false negative results. However, an increased sample volume could potentially yield signals even in case of low recoveries.

2.2. Storage

According to WBE approach the preferred storage temperature of the samples is −20°C in order to inactivate the bacterial activity and consequently the degradation of the CECs [12]. However, this temperature may not be suitable for the analysis of SARS-CoV-2 genetic material. Freezing and de-freezing the sample could lead to degradation of the genetic material of SARS-CoV-2 by the RNAses in wastewater [36]. This could result in loss of the viral load having detrimental effects on the results of the analysis. The lack of a commonly verified storage protocol led the various groups to use different storage temperatures (Table 1) which may have impact on the results and urgently needs to be addressed. It is preferable not to store the raw wastewater, but the concentrated one (see section 2.3) at − 80°C, where RNAses are not presented in the extract.

Another important factor is the storage duration before the analysis which significantly varies among the reviewed studies (Table 1). Although, the SARS-CoV survives for two days at 20°C and 14 days at 4°C in both hospital and domestic wastewater [37], the fate of SARS-CoV-2 in wastewater is still unclear.

2.3. Concentration step

Nowadays, various concentration methods are used for the enrichment of SARS-CoV-2 in wastewater (Table 1). Although these methods have been successfully applied for the recovery of non-enveloped enteric viruses from wastewater, such as polioviruses, adenoviruses, noroviruses, and enteroviruses [38,39], the recovery rates for enveloped viruses, such as SARS-CoV-2, are not yet satisfactory. An important drawback of the present methodology as it has been described was the lack of ideal external control standards that could have the same properties as the SARS-CoV-2. For instance, Deuterated and/or ^{13}C surrogates are used for quantification of the target compounds in traditional WBE.

Ahmed et al. [28], compared seven concentration methods and estimated the recovery of SARS-CoV-2 in wastewater. It was suggested that absorption-extraction methods can provide rapid and straightforward recovery of SARS-CoV-2 in wastewater. The best recovery in electronegative membrane was observed by the addition of $MgCl_2$ to the wastewater prior to filtration. In ultrafiltration, the Amicon Ultra-15 filter showed satisfactory performance. However, recoveries depended on the sample analysis volume and the filter type. Finally, PEG showed relatively poor yields resulting in co-concentration of PCR inhibitors. Pre-centrifugation that is commonly used in concentration, to remove larger particles and debris, could affect the recovery, since generation of pellet could absorb part of the genetic material.

It is worth noting that only a small number of data are available for enveloped viruses, highlighting the need for much further investigation of the existing methods and their recovery efficiencies between different water types, as well as the elucidation of novel approaches.

2.4. Isolation of viruses in wastewater

Isolation of RNA in the concentrated extracts is usually performed by commercially available kits (Table 1). These kits are designed to isolate RNA from bacteria, algae and fungi combining lysis and inhibitor to yield high-quality, ready-to-use RNA, even with highly complex matrices. Then RNA can be used in different detection techniques and other downstream applications. Several external controls like Pseudomonas bacteriophage Φ6 and Hep G Armored RNA have been used as a sample process control in order to determinate the efficiency of RNA extraction and to check the PCR inhibition [21,34].

The yield of SARS-CoV-2 virus recovered during concentration and RNA isolation must be determined. A non-target RNA sequence need to be added in all tested samples as exogenous spiked-in control either after the concentration step or after addition of the lysis solution to allow the estimation of sample to sample variation in the viral extraction procedure [20]. Recovery of exogenous

Table 1
Reviewed studies of the five steps of analysis.

Sampling	Analysis Volume	Storage conditions	Concentration	Isolation	Detection	External Control	Recovery (%)	Cq	Ref
Grab (2 L)	100–200 mL	4°C for 24 h	• Adsorption-extraction (pH = 4; neutral pH; 25 mM $MgCl_2$) • Centrifugal filter (Amicon ultra-15 (30 K); Centricon Plus-70) • Polyethylene glycol (PEG 8000) precipitation • Ultracentrifugation	RNeasy PowerMicrobiome Kit (slightly modified)	TaqMan-based RT-qPCR	Oncorhynchus keta (PCR inhibitors)	NA	19-120 copies/L Cq: 37.5–39	[28]
24 h composite and grab	50 mL	4°C	• Electronegative membranes • Ultrafiltration	Combination of two kits (RNeasy PowerWater Kit and RNeasy PowerMicrobiome Kit)	RT-qPCR; Target sequencing (Illumina and Sanger)	Murine hepatitis virus (Recovery) Oncorhynchus keta (PCR inhibitors)	26.7–65.7	NA	[19]
Weekly pooled 24 h and grab	125 mL (Innovaprep) 100 mL (Electronegative filtration)	Immediately concentrated	• InnovaPrep Concentrating Pipette Select • Electronegative filtration	Armored RNA Quant (protocol B 2.0.1 with modifications)	Reverse transcription droplet digital PCR (RT-ddPCR)	Bovine coronavirus (BCoV) (Recovery) Bovine respiratory syncytial virus (BRSV) (Recovery) Hep G Armored RNA (PCR inhibitors)	• 45–73 (no conc, BCoV) 62–88 (no conc, BRSV) • 3.1–7.6 (Innovaprep, BCoV) • 4.6–10.6 ((Innovaprep, BRSV) • 2.0–7.6 (electronegative filtration, BCoV) • 2.8–10.4 (electronegative filtration, BRSV)	10^2-10^5 copies/L	[29]
Grab	200–5000 mL	Immediately analyzed	• Electronegative membrane-vortex • Filtration with mixed cellulose-ester membrane	QIAamp Viral RNA Mini Kit and RNeasy PowerWater Kit	RT-qPCR, Nested PCR and Direct nucleotide sequencing using the Sanger method	Coliphage MS2	• 46.4–96.8 (electronegative membrane-vortex) • 4.8–12.2 (adsorption direct RNA extraction methods)	EMV method: 1.8×10^5–1.0×10^8 copies/L Adsorption-direct RNA extraction method: <1.0×10 –1.3×10^6 copies/L	[24]
Grab and pool	50 mL	19 days at 4°C and others Immediately analyzed	• PEG 9000	NucleoSpin® RNA Virus	TaqPath™ Covid-19 RT-PCR Kit	Coliphage MS2	57 (geometric mean)	32.45-39.56 5.6×10 - 3.5×10^2 copies/L	[30]
24 h composite	250 mL	−20°C	• PEG-dextran method	NucliSENS miniMAG semi-automated extraction system	Three different nested RT-PCR assays, one real-time qPCR assay and direct nucleotide sequencing using the Sanger method	Alphacoronavirus HCoV 229E	1.34-2.74	10^2 -5.6×10^4 copies/L	[23,31]
24 h flow-dependent composite	250 mL	4°C for 24 h	• Centricon® Plus-70 centrifugal ultrafilters	RNeasy PowerMicrobiome Kit	RT-qPCR	F-specific RNA phages Dengue virus	• 8.1–52.7 (Dengue virus internal control) • 23–123 (culturable F-specific RNA phages)	2.6-2.2×10^3 copies/L Cq: 29.9–39.2	[20]
24 h composite (time or flow dependent)	500 mL	5 ± 3°C until analysis within 48 h	• Direct flocculation	NucliSENS® miniMAG® system	EliGene COVID19 Basic A RT kit	Transmissible gastroenteritis coronavirus (TGEV)	22.5–48.6	Cq: 34-40	[32]
Grab (0.2 L)	200 mL				RT-qPCR	Mengovirus vMC0	2.56-18.9	Cq: 33.87–37.84	[33]

(*continued on next page*)

Table 1 (*continued*)

Sampling	Analysis Volume	Storage conditions	Concentration	Isolation	Detection	External Control	Recovery (%)	Cq	Ref
		4°C for almost two months	• Aluminum-driven flocculation	Centrifugation at 1900×g for 30 min				1.6–9.7 × 10^5 copies/L	
Grab (0.5–1 L)	200 mL	4°C and concentrated within 24 h	• Aluminum hydroxide adsorption-precipitation protocol	Nucleo-Spin RNA virus kit	TaqMan real-time RT-PCR	Mengovirus vMC0	8.9–13.1	6.3 × 10^4 -6.3 × 10^5 copies/L Cq: 34.68–40	[22]
Grab (1 L)	50 mL	Not available	• No concentration performed	QIAMP Viral RNA mini kit	RT-qPCR, whole genome sequencing and Ion Torrent PGM	Internal plasmidic control included in the 2019-nCoV real-time RT-PCR kit panel	>90	Cq: ≤40	[34]
24-h composite and grab	250 mL	−80°C until analysis and within 6 h	• Ultrafiltration • Centricon® Plus-70 centrifugal filter • Electronegative membrane	ZR Viral RNA Kit	RT-qPCR	Pseudomonas bacteriophage Φ6	• 56 (Centricon Filter) • 54 (Electronegative mebrane)	3.1–7.5 × 10^3 copies/L	[21]
24 h flow-dependent composite	45 mL	−18°C	• Ultrafiltration	NucleoSpin RNA Virus kit	RT-qPCR and Sanger sequencing analysis	NA	NA	30-200 gene equivalents/L	[35]

control in each case should be estimated with respect to the levels of an equivalent amount of exogenous control that was added to the wastewater (representing 100% recovery).

2.5. Detection of SARS-CoV-2

Numerous research groups have published specific assays for virus detection (Table 1). However, the sensitivity and specificity of these methods are quite diverse, due to different target genes that are amplified and various pairs of specific primers and probes (Table 2). The most commonly used primers and probes are the ones suggested by Centers for Disease Control and Prevention (CDC). Besides, the false negative and false positive rates are relatively high due to possible errors in the sampling and testing procedure [4]. Low viral loads are contained in wastewater and in combination with the potential degradation of the RNA may limit the performance of the assay. The first SARS-CoV-2 PCR assays were developed against the E-gene and the N-gene to detect the virus in wastewater for SARS-CoV-2 surveillance [20–22,24]. To a minor proportion, also PCR targeting SARS-CoV-2 RdRP and ORF1ab was used [23,30,31]. The variation of targeted sequences that were amplified by PCR reaction could explain the range of the positivity rates. As the genetic material of SARS-CoV-2 is degraded in the wastewater samples, more than one target genes should be detected to limit false positive or negative results.

The presence of SARS-CoV-2 in wastewater samples, has already been confirmed using the "gold standard" RT-qPCR by the detection of specific viral RNA sequences (Table 1). The currently applied assays of RT-qPCR for the detection of SARS-CoV-2 in wastewater samples are based on one-step RT-qPCR allowing efficient cDNA synthesis and Real-Time PCR in a single tube. Virus-specific primers and probes are designed to target at least two regions of the virus [40].

Several RT-qPCR kits are currently commercially available and some of these have already obtained CE-IVD certification in order to be used for routine testing of COVID-19 diagnosis [41,42]. Vogels et al., compared the analytical efficiency and sensitivity among four RT-qPCR assays in order to evaluate the performance of primer-probe sets. The results revealed that although all primer-probe sets were capable to identify SARS-CoV-2, however significant discrepancies in analytical sensitivity were demonstrated in cases where the viral load was very low [43]. Although these kits have gained wide acceptance in clinical science, could be also used in WBE.

The main drawback of PCR based assays remains the false-negative results that range between 20 and 40% depending on the viral load and the clinical performance of each assay to assess the gradual decline of viral burden during the follow-up of quarantined patients [44]. Few groups used sequencing technology in wastewater analysis to confirm the RT-qPCR specificity [19,34,35].

Ultrasensitive assays based on PCR (Nested PCR and droplet digital PCR (ddPCR)) were proven to be applicable methods for WBE (Table 1). Nested PCR provides enhanced sensitivity by reducing the non-specific binding with the use of two sets of primers, detecting very low amounts of viral load contained in wastewaters. ddPCR gaines popularity as a novel approach to nucleic acid quantification as it improves the limit of detection and accuracy in the detection of SARS-CoV-2 [45].The major benefit of ddPCR over qPCR is the direct absolute quantification of virus genome copy numbers in a sample without the necessity of external calibration. Comparison study between RT-qPCR and ddPCR with multiple primer/probe sets showed a significant improved performance of ddPCR in samples with low levels viral load [46]. However, ddPCR is still expensive, as specific and expensive instrumentation and high cost reagents are required.

Table 2
Target genes, primers and probes commonly used for analysis of SARS-CoV-2 in wastewater by PCR.

Target	Primers and probes Sequences (5-3)	References
N- Sarbeco protein	F-CACATTGGCACCCGCAATC R-GAGGAACGAGAAGAGGCTTG P-FAM-ACTTCCTCAAGGAACAACATTGCCA-BHQ1	[19,24]
THREE regions of nucleocapsid (N) gene (CDC)	2019-nCoV_N1−F: GAC CCC AAA ATC AGC GAA AT 2019-nCoV_N1-R: TCT GGT TAC TGC CAG TTG AAT CTG 2019-nCoV_N1−P: FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1 2019-nCoV_N2 Forward Primer TTA CAA ACA TTG GCC GCA AA 2019-nCoV_N2 Reverse Primer GCG CGA CAT TCC GAA GAA 2019-nCoV_N2 Probe FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1 2019-nCoV_Forward Primer: AGA TTT GGA CCT GCG AGC G 2019-nCoV_Reverse Primer: GAG CGG CTG TCT CCA CAA GT 2019-nCoV_F Probe: FAM − TTC TGA CCT GAA GGC TCT GCG CG − BHQ-1	[20−22,24]
ORF1ab	FW1 GTGCTAAACCACCGCCTG REV1 CAGATCATGGTTGCTTTGTAGGT FW2 CGCCTGGAGATCAATTTAAACAC REV2 ACCTGTAAAACCCCATTGTTGA	[23,30,31]
RdRP	RdRP_SARSr-F2 GTGARATGGTCATGTGTGGCGG RdRP_SARSr-R2 CARATGTTAAASACACTATTAGCATA RdRP_SARSr-P2 FAM-CAGGTGGAACCTCATCAGGAGATGCBHQ1	
S	WuhanCoV-spk1-F: TTGGCAAAATTCAAGACTCACTTT WuhanCoV-spk2-R: TGTGGTTCATAAAAATTCCTTTGTG	
Envelope protein (E) gene	E_Sarbeco_F: ACAGGTACGTTAATAGTTAATAGCGT E_Sarbeco_R: ATATTGCAGCAGTACGCACACA E_Sarbeco_PROBE: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	[20]

Sequencing methods are used for the confirmation of the PCR products. Since wastewater is a complex matrix and environmental contaminants could affect PCR. Few groups used this approach to eliminate false positive results [19,23,24,31,35]. For instance, Westhaus et al. clearly pointed out the necessity for a confirmation of positive RT-qPCR results by sequencing or other appropriate techniques in order to avoid false positive results. They noticed that positive signals in RT-qPCR were not confirmed by sequencing for the retained samples from 2017 and 2018 [35]. Although sequencing could not support the massive population screening or even the testing of symptomatic and suspicious cases, due to high cost and limited number of laboratories with the experienced staff and the required organology, next generation sequencing (NGS) could be applied in WBE. In this regard, the number of WBE-related samples is not a deterrent for NGS-based analysis, and most importantly, the sequencing of the entire SARS-CoV-2 genome will clearly benefit population diagnostics and the study of viral/pandemic evolution. Overall, sequencing is still under exploited in the field of WBE.

Loop-mediated isothermal amplification (LAMP) is another simple, fast (roughly an hour) and sensitive isothermal amplification technique, that is less dependent on specific equipment, and has been widely used for the development of point of care (POC) techniques for the detection of viruses and other pathogens in field and/or resource-limited settings. Targeting the viral RNA of SARS-CoV-2 in more regions (orf1ab, S gene and N gene) using four sets of LAMP primers (6 primers in each set) the sensitivity of LAMP assay could be 80 copies of viral RNA per mL in a sample [47]. More recently, a multiplex LAMP (mRT-LAMP) coupled with a nanoparticle-based lateral flow biosensor (LFB) assay (mRT-LAMP-LFB) was developed for diagnosing COVID-19. This assay is characterized with high analytical sensitivity and specificity (100%), whereas the limit of detection (LOD) is 12 copies (for each detection target) per reaction [1]. Therefore, LAMP could have applicability in WBE because of the low viral loads in wastewater.

Highly sensitive cell-based biosensors-tests have been developed in order to be used for the detection of SARS-CoV-2 in a matter of minutes [48]. Mao et al., have proposed a paper-based device as an early warning system for WBE, since they are easy to stack, store, and transport. In addition, paper-based devices can also be incinerated after use, and thus reduce the risk of contamination [49,50]. Sensors can potentially be used as a pre-screening and/or supplementary tool to reach the objective of WBE, monitoring of SARS-CoV-2 in a community level [50].

3. Conclusions and future perspectives

Wastewater-based epidemiology was proved to be a powerful and cost-effective tool for the investigation of the lifestyle exposure and health status of a population. This approach could provide real-time data and act as an early warning system to assist authorities make decisions, such as interventions, quarantine and lockdown to prevent the spread of an epidemic disease. Monitoring of SARS-CoV-2 in wastewater has gained rapidly popularity, but there are still various limitations and challenges to be addressed in the analytical protocols. Taking under consideration all the available peer-reviewed studies, a few suggestions could be proposed for each of the five steps of the protocol.

- 24 h composite samples are highly recommended to assure the representativeness of the sampling.
- Designing a daily sampling campaign could be useful to evaluate the trends before, during and after the interventions (i.e. lockdown, restrictions in activities).
- It is crucial storing the samples at fridge temperature (4°C) and perform analysis as soon as possible.
- Concentration should not be skipped in order to enrich the viral loads in wastewater and achieve higher sensitivity.
- External controls are necessary to evaluate the recovery (trueness) and correct sample to sample variation.
- RT-qPCR is the most widely used instrumental method for the detection of SARS-CoV-2, because of its high sensitivity, high throughput, laboratory availability and cost-effectiveness.
- Other instrumental techniques such as ddPCR, nested PCR and target sequencing could be further exploited by WBE.
- CRISPR is proposed as a powerful tool on RNA detection, since it is fast, easily operated and accurate.

- LAMP could be further investigated, since it is less dependent on specific equipment and has been widely used for the development of point of care techniques for the detection of viruses.
- Biosensors is an emerging proposed tool for on-site and real-time monitoring of wastewater with clear advantages such as cost effectiveness, rapidness and easily applicable by non-experts

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 presented in this paper.

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