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Review

Tracing COVID-19 Trails in Wastewater: A Systematic Review of SARS-CoV-2 Surveillance with Viral Variants

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Abstract: The emergence of new variants of SARS-CoV-2 associated with varying infectivity, pathogenicity, diagnosis, and effectiveness against treatments challenged the overall management of the COVID-19 pandemic. Wastewater surveillance (WWS), i.e., monitoring COVID-19 infections in communities through detecting viruses in wastewater, was applied to track the emergence and spread of SARS-CoV-2 variants globally. However, there is a lack of comprehensive understanding of the use and effectiveness of WWS for new SARS-CoV-2 variants. Here we systematically reviewed published articles reporting monitoring of different SARS-CoV-2 variants in wastewater by following the PRISMA guidelines and provided the current state of the art of this study area. A total of 80 WWS studies were found that reported different monitoring variants of SARS-CoV-2 until November 2022. Most of these studies (66 out of the total 80, 82.5%) were conducted in Europe and North America, i.e., resource-rich countries. There was a high variation in WWS sampling strategy around the world, with composite sampling (50/66 total studies, 76%) as the primary method in resource-rich countries. In contrast, grab sampling was more common (8/14 total studies, 57%) in resource-limited countries. Among detection methods, the reverse transcriptase polymerase chain reaction (RT-PCR)-based sequencing method and quantitative RT-PCR method were commonly used for monitoring SARS-CoV-2 variants in wastewater.

Among different variants, the B1.1.7 (Alpha) variant that appeared earlier in the pandemic was the most reported (48/80 total studies), followed by B.1.617.2 (Delta), B.1.351 (Beta), P.1 (Gamma), and others in wastewater. All variants reported in WWS studies followed the same pattern as the clinical reporting within the same timeline, demonstrating that WWS tracked all variants in a timely way when the variants emerged. Thus, wastewater monitoring may be utilized to identify the presence or absence of SARS-CoV-2 and follow the development and transmission of existing and emerging variants. Routine wastewater monitoring is a powerful infectious disease surveillance tool when implemented globally.

Keywords: COVID-19; SARS-CoV-2 variants; Alpha (B.1.1.7); Delta (B.1.617.2); Omicron (B.1.1.529); wastewater-based epidemiology

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), continuously underwent mutations leading to the emergence of new variants [1]. These variants are of great concern [2–4], as they might be associated with increased infectivity [1,5], severity [1,6,7], could have higher shedding rates [8], the potential to escape natural or vaccine-induced immunity [9,10], and can also affect the performance of diagnostic methodologies [11,12]. Such changes in virus characteristics affected the overall management plan for the COVID-19 pandemic. For example, it led to travel restrictions both locally and internationally for people from infected areas [1,7], and many more consequences on the daily lives of individuals. Therefore, the emergence of SARS-CoV-2 variants increased the need for genomic surveillance and other innovative tools to protect public health.

Whole-genome sequencing (WGS) of clinical specimens is a primary approach for identifying new emerging variants [13], by comparing the sample genome with the reference genome [14]. However, using WGS for monitoring each clinical specimen is time-consuming, labor-intensive, and expensive, and is usually conducted for individuals with clinical symptoms. Many of the COVID-19-infected individuals can be asymptomatic, so only relying on a clinical monitoring approach in the surveillance can miss the mutant variants carried by asymptomatic individuals.

Wastewater surveillance (WWS), also known as wastewater-based epidemiology (WBE), of infectious diseases through analyzing municipal sewage proved to be a cost-effective approach for monitoring the circulation of SARS-CoV-2 at a population level, covering both symptomatic and asymptomatic individuals [15–20]. In contrast to the clinical approach, WWS is a comprehensive, rapid technique for regular monitoring and tracking of the possible emergence of new variants at a population level [19–23]. From a surveillance point of view, municipal raw sewage can be a good material for SARS-CoV-2 monitoring, as it comprises the entire population of a community, both healthy and infected individuals (symptomatic, asymptomatic, pre-symptomatic, and post-symptomatic), contributing through feces, nasal mucus, and sputum to sewage from households, hospitals, and nursing homes [16,17,24]. Globally, many studies reported monitoring different variants of SARS-CoV-2 in wastewater [11,15–17,20,24–28], thereby highlighting WWS as an alternative tool for detecting different variants in communities. However, a comprehensive evaluation of the state-of-art use of WWS for monitoring SARS-CoV-2 variants is lacking. Such data can help evaluate and optimize WWS for monitoring SARS-CoV-2 variants. Such information can also be useful in managing future infectious outbreaks, such as how the wild and mutated variants differ among geological locations. Here, this review provides a comprehensive evaluation of the use of WWS for monitoring emerging SARS-CoV-2 variants, considering the opportunities and limitations of different methods used to analyze variants and the corresponding results (Figure 1).

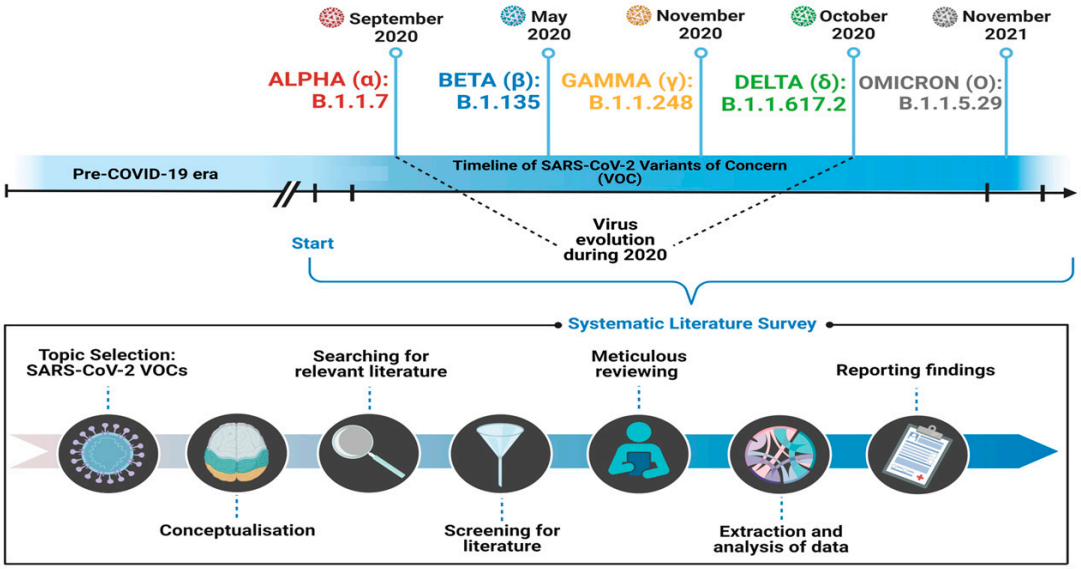

Figure 1. Conceptual framework of the study.

2. Theoretical Background: The Emergence of SARS-CoV-2 Variants

SARS-CoV-2 is an enveloped single-strand RNA (ssRNA) virus belonging to the *Coronaviridae* family and genus *Betacoronavirus* [9,29]. As with other ssRNA viruses, SARS-CoV-2 contains RNA-dependent RNA polymerase (RdRP), which is responsible for sub-genomic mRNA synthesis for producing viral proteins, including the virus envelope and spike proteins [30]. RNA viruses are relatively prone to adapt more rapidly to a changed environment by changing their genome structure.

SARS-CoV-2 continuously evolves into new variants due to genetic mutation and viral recombination [1,2,13,31]. Mutation refers to at least a single change in a virus's genetic code. Genetic modifications can change the virus's characteristics [1]. A SARS-CoV-2 variant can have one or more mutations that differentiate its features from other variants. SARS-CoV-2 has a similar mutation mechanism to other ssRNA viruses that lack proofreading capability, giving rise to new variants [25]. Uncorrected mutations occur during genome replication, recombination, and RNA editing by the deaminase of the infected host [13]. A recombinant variant is created due to a combination of genetic material from two different variants, and a mutant variant is created due to a mutation in RNA. A lineage is a group of closely related viruses with a common ancestor [32]. The ancestral SARS-CoV-2 (wild variant) genome evolved into several lineages (https://cov-lineages.org/lineage_list.html, accessed on 28 November 2022), such as the Alpha (B.1.1.7), Delta (B. 1.617.2), and Omicron (B.1.1.529) [2,3,7,11,28,32–37], due to exposure to some selective pressure [38]. Most of these new variants were developed due to viral spike protein (S-protein) mutation [39].

2.1. Alpha (B.1.1.7 and Q Lineages)

The Alpha variant was first isolated in the United Kingdom in September 2020 and was followed by an upsurge in infection in December 2020 [40]. Soon after, it became the dominant variant until August 2021 in many countries, including the US, India, Sweden, and globally in at least 189 countries (Table 1). The World Health Organization (WHO) classified the Alpha variant as a variant of concern (VOC) on 29 December 2020 [10], after rising hospitalization cases and creating a strain on the public health system and facilities across countries [41]. The Alpha variant was reported to be about 100-fold more lethal than the original SARS-CoV-2 strain [6]. Further, mRNA vaccines were reported to be about 68% less effective against this variant [6]. On 21 September 2021, the WHO designated the Alpha variant as the "variant being monitored" [1,7]. After 2022, this variant's circulation drastically reduced worldwide, following the emergence of Delta variants, probably due to the impact on vaccine-induced immunity (Table 1).

Table 1. SARS-CoV-2 variants and lineages [1,7,33].

WHO Label/Pango Lineage	Country First Detected	Spike Mutations of Interest	Outbreak Countries	Major Outbreak Peaks	Classification (WHO) during November 2022	Outbreak Condition during November 2022
Alpha/B.1.1.7	United Kingdom, September 2020	N501Y, D614G, P681H	At least 189 countries, predominant in the US, India, Sweden, France, Spain, Australia, Nigeria, and so on (1.2 million cases globally reported).	November 2020 to August 2021	VOC: 29 December 2020 VBM: 21 September 2021 PVOC: 9 March 2022	Drastically reduced circulation globally, with almost no reporting at the time of writing the manuscript.
Delta/B.1.617.2	India, October 2020	L452R, T478K, D614G, P681R	At least 208 countries, predominant in the US, UK, Japan, Italy, India, Germany, Canada, Denmark, France, and so on (4.4 million cases globally).	May 2021–January 2022	VOC: 15 June 2021 VBM: 14 April 2022, PVOC: 7 June 2022	Abundance is very low at the time of writing the manuscript.
Beta/B.1.351	South Africa, May 2020	K417N, E484K, N501Y, D614G, A701V	At least 127 countries, predominant in South Africa, the US, India, Sweden, France, Spain, Australia, Nigeria, Iran, and so on (43,000 cases globally).	November 2020 to August 2021	VOC: 29 December 2020 VBM: 21 September 2021, PVOC: 9 March 2022	Drastically reduced circulation globally, with almost no reporting at the time of writing the manuscript.
Gamma/P.1	Brazil, November 2020	K417T, E484K, N501Y, D614G, H655Y	At least 93 countries, predominant in the US, Canada, Brazil, Argentina, Chile, Italy, Peru, Mexico, Sweden, South Korea, Venezuela, and so on (74,300 cases globally).	Feb 2021–November 2021	VOC: 29 December 2020 VBM: 21 September 2021 PVOC: 9 March 2022	No longer detected or detected at extremely low levels globally.
Epsilon/B.1.427, B.1429	California USA, July 2020	I4205V and D1183Y in the ORF1ab gene, and S13I, W152C, L452R in the spike protein's S-gene	At least 45 countries.	November 2020–March 2021	VOC, March 2021. VBM-September 2021. Previously circulating VOI: March 2022 (WHO),	After an initial increase, its prevalence rapidly decreased from February 2021 and was outcompeted by the more transmissible Alpha variant.
Lambda/C.37	Peru, August 2020	Virus's spike protein code: G75V, T76I, Δ246–252, L452Q, F490S, D614G and T859N	At least 45 countries (predominant in Peru, Chile, US). Total global cases of less than 10,000.	November 2020–November 2021	VOI June 2021	No longer reported.
Omicron/BA.2, BA.4, BA.5, BA.2.75, BQ.1, XBB	South Africa and Botswana November, 2021	BA.2 (y*), BA.4 (L452R, F486V, R493Q), BA.5 (L452R, F486V, R493Q). BA.2.75 (z**), BQ.1 (K444T, N460K), XBB (N460K, F490S)	At least 208 countries, predominant in the US, UK, Denmark, Canada, India, Japan, Germany, France, and so on (6.2 million cases globally, 14 December 2022).	November 2021-**Currently circulating lineages:** BA.2, BA.4, BA.5, and their recombinants and sub-lineages	BA.2, BA.4, and BA.5 are VOC, and BA.2.75, BQ.1, XBB are current VOI at the time of drafting the manuscript, December 2022.	Many lineages are currently going around the world (83,046 in the last four weeks of 14 December 2022).

Notes: Other variants monitored earlier: Eta/B.1.525 (VOI on 02/29/21, but VBM since 09/21/21), IOTA/B.1.526 (VOI on 02/29/21, but VBM since 09/21/21), Kappa/B.1.617.1 (VOI on 05/07/21 but VBM since 09/21/21), B.1.617.3 (VOI on 05/07/21, but VBM since 09/21/21), Zeta/P.2 (VOI on 02/26/21, but VBM since 09/21/21), and Mu/B.1.621 and B.1.621.1 (VBM on 09/21/21but VBM since 09/21/21) *Key: VOI—Variant of interest; VOC—Variants of* concern; *VBM—Variant being monitored; PVOC-Previous variant of concern.* y*: G142D, N211I, Δ212, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, S477N, T478K, E484A, Q493R, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K, z**: W152R, F157L, I210V, G257S, D339H, G446S, N460K, and Q493 (reversion).

2.2. Delta (B.1.617.2 and AY Lineages)

The Delta variant was first detected in India in October 2020 [10,42], and it swept rapidly through India and then the United Kingdom by mid-April 2021 before spreading to the US and the rest of the world [42]. The Delta variant was reported to be 60% more infectious and lethal than the Alpha variant [1]. It was reported that a single and double dose of AstraZeneca vaccine was 33% and 60% effective in reducing the Delta lineage infection, respectively, compared to 60% and 66% on the Alpha lineage [43,44]. Similar results (i.e., less effective vaccine than with Alpha lineage) were observed in a study when Pfizer vaccines were administered [42]. The emergence of this variant caused a delay in

the United Kingdom's reopening plans after several months of lockdown beyond June 2021 [9]. It became a VOC in the US on 15 June 2021 [1], after it became evident that people infected with the Delta variant were twice as likely to become hospitalized than those with the Alpha variant [41]. This implied that the Delta variant exhibited more infectivity than earlier variants [42]. Until October 2021, Delta was the most dominant variant in the world, with about 90% sequences in the Global Initiative on Sharing Avian Influenza Data (GISAID).

2.3. Omicron (B.1.1.529 and BA Lineages)

The Omicron variant was declared a VOC immediately after it was reported in South Africa in November 2021 [1]. The Omicron variant has the highest number of mutations, compared to the reference wild SARS-CoV-2 genome, with 37 mutations in the spike (S) protein, three mutations in the nucleocapsid (N) protein, one mutation in the envelope (E) protein, three mutations in the membrane (M) protein, and 10 synonymous mutations [45]. This variant is more contagious than the earlier variants, with a reported rise of cases from hundreds per day to thousands per day in South Africa over two weeks [9]. It soon began to spread to several other countries and became one of the most dominant variants after December 2021 [25,46,47]. A subvariant known as BA.2 was also discovered and monitored as it accounted for 23% of cases in the US as of March 2022 [48]. The Omicron BA.2 sub-variant has a mutation on the spike protein, which is responsible for infecting host cells, thereby increasing infectability and having the capacity to evade immunity [49], most especially those who recovered from previous COVID-19 variants infection but were yet to be vaccinated [50]. The Omicron variant and its sub-lineages were the most dominant variants circulating globally in 2022, for more than 98% of sequences shared on GISAID between February 2022 and November 2022 belonging to Omicron. Omicron is a complex variant that continues to evolve, leading to descendent lineages with different genetic constellations of mutations [7]. Despite its high transmissibility, it has lower severity than previous Delta and Alpha variants. In December 2022, the world was still passing through the pandemic of the Omicron variant. A total of 83,046 cases were reported in GISAID within four weeks (https://gisaid.org/hcov19-variants/, accessed on: 7 December 2022) (Table 1).

2.4. Other Variants

Aside from the variants mentioned above, many other variants were first declared as VOC but were later re-designated as variants of interest (VOI) [1,33] or declared as variants being monitored (VBM), previously circulating VOCs or VOIs or formerly monitored variants (FMVs). The designation of VOC, VOI, VBM, and FMVs are working definitions periodically updated by WHO and CDC (US and EU) (Table S1).

Among the emergence of different variants, the Beta (B.1.351) variant was detected in South Africa [51], and the Gamma (P1) variant was identified in Brazil in November 2020 [34,46]. Both lineages of Epsilon (B.1.427 and B.1.429) were identified in California [35] and were reported to have higher transmissibility, infectibility, and severity than the preceding variants and lineages [41]. The Lambda variant (lineage C.37) was first reported in Peru in August 2020 and was designated as VOI by WHO on 14 June 2021 [36]. This variant was reported to be more resistant to neutralizing antibodies than other variants [36]. The Lambda variant was suspected to be more resistant to vaccines than the Alpha and Gamma variants [37]. Table 1 shows details of SARS-CoV-2 variants and their WHO designation at the time of review. This highlights the importance of genetic surveillance of SARS-CoV-2 variants worldwide.

3. Methodology

A thorough literature search was conducted in November 2022 using ScienceDirect, Google Scholar, PubMed, Web of Science, Scopus, and NCBI databases. No publication date or language restrictions were applied during the search, and Booleans "AND" and

"OR" to combine keywords were used. Searches were directed toward the review objectives with pertinent keyword combinations (a) wastewater surveillance; (b) SARS-CoV-2 and emerging variants; (c) variants of concern in wastewater; (d) genomic tools for SARS-CoV-2 monitoring; (e) wastewater-based epidemiology for SARS-CoV-2 variants; (f) next-generation sequencing for SARS-CoV-2; (g) SARS-CoV-2 variants in wastewater; and (h) Alpha, Delta, and Omicron variants in wastewater. The literature search process is presented in Figure 2, following PRISMA guidelines [52]. The included literature was mostly peer-reviewed journal articles (except two pre-print). Sole clinical monitoring of SARS-CoV-2 variants not including WWS, methodology comparison studies without surveillance aim, review papers without original data, and numerical modeling papers were excluded. The database search identified 718 studies, as shown in Figure 2.

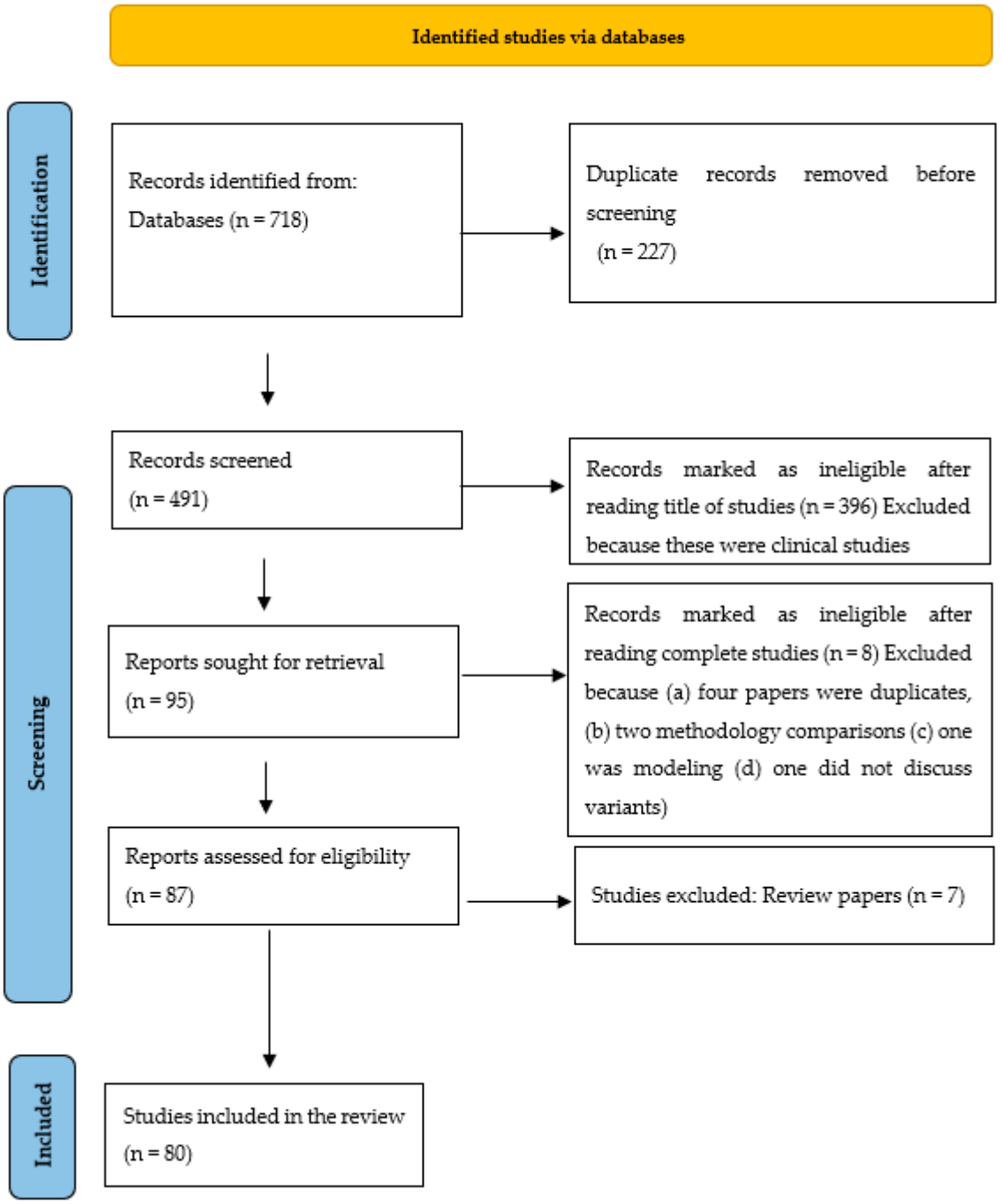

Figure 2. PRISMA flow diagram of literature search.

After the de-duplication steps, a total of 491 papers remained. Upon further screening of titles and abstracts, 396 research articles related to clinical testing were removed, leaving 95 articles for full-text screening. After reading 95 articles, an additional 15 articles were removed from the list as seven were found to be review papers: four were duplicates of earlier studies, two studies were only methodology comparisons, one was a modeling study, and one did not report variants. In total, 80 publications reporting SARS-CoV-2 variants in wastewater were included in this systematic review (Figure 2).

4. Results

4.1. Geospatial Distribution

The majority of the WWS studies (66/80, 82.5%) included in this review were conducted in resource-rich countries in Europe and North America (Figure 3). Out of the total of 80 studies, 38 (47.5%) were in Europe [4,9,18,23,28,53–83], 28 (35.0%) in North America (20 studies in the US [11,84–101], and 8 studies in Canada [102–109]), 10 studies (12.5%) in Asia [110–117], 2 studies in South America [118,119], and 1 study each were conducted in Africa [120] and Australia [121] (Table S1).

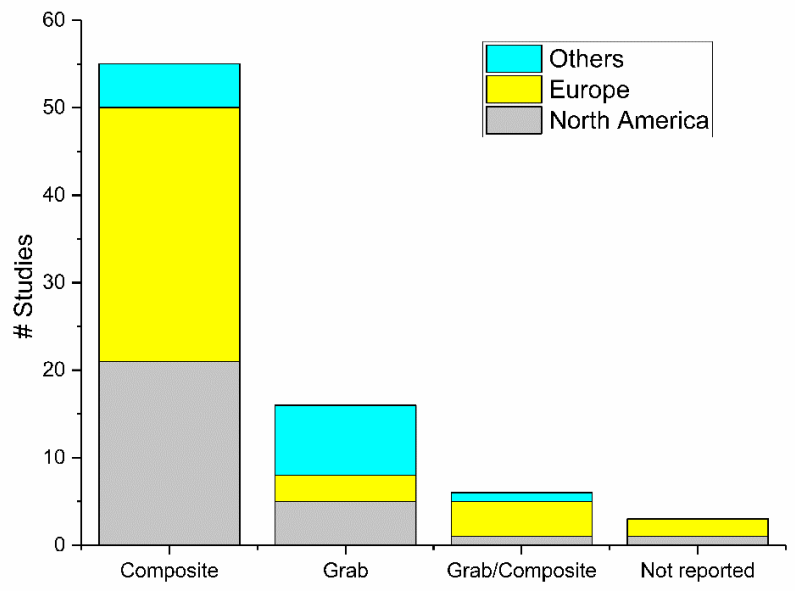

Figure 3. Number of studies based on sample collection type in different continents (others = countries from Asia, Africa, Australia, and South America).

4.2. Sampling Techniques

In general, autosamplers were used for taking composite samples that were either time proportional (equal aliquot is taken at a fixed time interval) or flow proportional (after a specific volume based on the flow rate of the source, an aliquot of sample is taken) [15]. Out of 80 studies, 55 (68.8%) used composite sampling, 16 (20%) used grab sampling, six (7.5%) used both composite and grab sampling, and three studies (3.8%) did not report the sampling technique used in their research. As shown in Figure 3, composite sampling was relatively more frequent in high-resource countries compared to other countries. Furthermore, the review found that resource-limited countries lack composite samplers due to the associated high costs and are most likely to rely on grab sampling (Figure 3). In resource-rich countries, grab samplers were primarily used in sub-catchments or facilities (such as hospital sewage or college campuses) or small regions of communities [18,57,101].

Out of 55 studies using composite sampling, 30 used time proportional, 14 studies used flow proportional, and the others used composite settled solids or did not report on sampling. In a previous report, proportional flow sampling was considered less biased than the time-proportional sampling mode [122]. These autosamplers have higher capital and operating (installation and maintenance) costs and may not be easily accessible in

low-resource settings. In a recent study, grab samples and 24 h composite samples showed comparable results during relatively low COVID-19 incidence [123]. Grab sampling is relatively easier and faster and does not need any automated equipment. It provides the status of SARS-CoV-2 only during the sample collection, so it is less representative of fecal community contributions than composite samples. Composite sampling is the collection of multiple grab samples, so it is more representative and provides the average situation of SARS-CoV-2 for a certain time interval. Such discrepancy in the sampling mode among resource-rich and resource-limited countries explains the lack of resources against emerging diseases or conditions, such as the COVID-19 global pandemic. Therefore, resource-limited countries deserve more global attention and funding for fighting emerging and re-emerging diseases.

4.3. Concentration Methods

Virus concentration is a critical step to detecting the low concentration of SARS-CoV-2 and its variants in wastewater. Our review noted a high variation in virus concentration methods. The most used methods were polyethylene glycol (PEG) precipitation, ultrafiltration, electro-negative membrane filtration, ultracentrifugation, aluminum hydroxide adsorption–precipitation, $AlCl_3$ precipitation, and skimmed milk (SM) flocculation. Many studies combined more than one concentration method and optimized it by using positive control during the extraction process. Among various virus concentration methods, PEG precipitation, skimmed milk (SM) flocculation, and aluminum polychloride (PAC) flocculation are reported to be relatively more cost-effective methods than ultrafiltration and ultracentrifugation [124]. The SM method can handle a large volume of samples at a time, it does not require special equipment, and the number of processing steps is relatively smaller than many other methods [124]. An earlier study reported that PEG precipitation and PAC flocculation were the most effective virus concentration methods from wastewater (62.2% and 45.0%, respectively) among eleven compared concentration methods [124].

4.4. Analytical Methods to Detect Variants

Among variant detection methods, PCR amplification-based sequencing was the dominant (49/80 of total studies) for SARS-CoV-2 variants analysis in wastewater. Among the various PCR-based amplification methods, including RT-PCR, five studies used nested PCR (nPCR), and multiplex tiling PCR was employed to prepare the cDNA and library for the downstream sequencing analysis of SARS-CoV-2 variants [4,23,102]. The workflow of sequencing-based detection methods includes three main steps. The first started with pre-screening of the total SARS-CoV-2 RNA by using the classical reverse transcriptase quantitative polymerase chain reaction (RT-qPCR assays targeting N1, N2, RdRP, and E genes) suggested by US CDC to screen SARS-CoV-2-positive samples. Then, positive samples with a Ct value (<35) were amplified by RT-PCR to generate a cDNA and sequencing library. The RT-PCR generating cDNA can also be a two-step approach, where cDNA synthesis is conducted separately and only after that PCR amplification takes place. Finally, high-throughput sequencing methods were employed to acquire sequence information on the samples, and the alignment between sample sequences and reference genomes was used to detect single-nucleotide variants (SNVs) or specific mutations associated with the variant/lineage.

Wurtz et al. also confirmed that direct sequencing is more accurate than the RT-PCR method in detecting SARS-CoV-2 variants [83]. However, according to another report (Volz et al., 2021), it is an instrument of high cost and requires some technical skills [125]. The sample size must also be certain to give an acceptable result. Hence, it was advised by Vo et al. (2022) that other less costly methods, such as the TaqMan RT-PCR, characterize the strain before using the WGS to confirm [11].

The RT-qPCR/RT-digital PCR (dPCR)-based method was the next most widely used detection method (31/80 of total studies) after the sequencing-based method among all 80 studies included in this study. RT-PCR-based quantitative detection methods (RT-qPCR

and RT-dPCR) could generate relative or absolute quantification results that can directly reveal the concentration of SARS-CoV-2 RNA in wastewater associated with clinical data to enhance the monitoring of SARS-CoV-2 prevalence in communities [126]. Thus, it became one of the most sensitive methods for monitoring SARS-CoV-2 RNA in wastewater [127]. However, compared with sequencing methods, the RT-qPCR method has a certain lag in discovering the emergence of new variants because it requires a specific primer–probe design according to the details of the genomic information of new variants reported by clinical tests [126]. In addition, the gene targets of RT-qPCR/RT-dPCR methods were limited by fluorophores and the detection instrument. Thus, it is mainly adopted for detecting and quantifying only the already known variants circulating in communities or elsewhere.

Multiplexed RT-qPCR /RT-dPCR methods exhibited great potential in monitoring SARS-CoV-2 variants in wastewater, since they can be regarded as a low-cost replacement of sequencing methods to increase the output of RT-qPCR methods and achieve the quantification of new variants [126]. Peterson et al. developed an RT-qPCR allelic discrimination assay that was sufficiently sensitive and specific to achieve the detection and relative quantitation of SARS-CoV-2 variants (B.1.1.7, B.1.351, and P.1) in wastewater [109]. Caduff et al. also developed a drop-off RT-dPCR assay to analyze temporal dynamics of SARS-CoV-2 signature mutations (spike Δ69–70 and ORF1a Δ3675–3677) in wastewater [70]. Furthermore, Boogaerts et al. successfully optimized, validated, and applied a multiplex digital polymerase chain reaction (dPCR) assay to measure the emerging SARS-CoV-2 variants of concern (VOC) in influent wastewater [76]. All these methods successfully distinguished between SARS-CoV-2 RNA originating from the wild-type and B.1.1.7, B.1.351, P.1, and B.1.617.2 variants, thus having great potential in the application of SARS-CoV-2 monitoring in wastewater. In addition to enabling the detection and absolute quantification of various SARS-CoV-2 variants simultaneously, the multiplexed RT-qPCR methods could also reveal the proportion of new variants out of all prevalent strains in wastewater [97]. Through the relative quantification results acquired by simultaneously detecting the highly conserved sequence of SARS-CoV-2 genomes and specific genes of various new variants, multiplexed RT-PCR methods might be further used to provide insightful information on the proportion of new variants in communities. Thus, the various methods used by different studies reviewed have both advantages and disadvantages.

4.5. Detected Variants

Out of the total of 80 studies, the B.1.1.7 (Alpha) variant was the most targeted and detected variant (48/80 of total studies), followed by B.1.617.2 (Delta, 24/80 total studies), B.1.351 (Beta, 19/80 total studies), P.1 (Gamma, 19/80 total studies), and others in wastewater (Figure 4). As all variants reported as WWS followed the same pattern as the clinical reporting of variants in the same timeline, this demonstrated that WWS tracked all variants timely when they were developed (Table S1). The reason why the B1.1.7 (Alpha) variant was the most reported is that it appeared earlier than other variants in the pandemic, and thus during the gathering of papers for the present review (November 2022), there was more time to report the Alpha variant findings than findings of the other variants. Although Omicron is the largest among the different variants and constituted an ongoing pandemic at the time of writing this review, its importance was not yet reflected in the number of publications available by November 2022, being only the fifth most reported (Figure 4). This may be due to reporting delays.

In 49 sequencing-based analyses, 28 studies detected the B.1.1.7 (Alpha) variant, 20 studies detected the B.1.617.2 (Delta) variant, 15 detected the P.1 (Gamma) variant, 15 detected B.1.351 (Beta) variant, 7 detected various lineages Omicron (e.g., BA.1 and BA.2), and 7 other studies reported only other uncommon variants (Table S1). Similarly, out of 31 RT-qPCR/RT-dPCR methods-based studies, 20 detected the B.1.1.7 (Alpha) variant by targeting the △HV69/70 deletion, the N501Y mutation, or/and the N-D3L mutation. Nine studies detected the B.1.617.2 (Delta) variant by targeting SΔ157 mutation [70,76,109].

Six studies detected the B.1.351 (Beta) variant by targeting the N501Y mutation, ORF1a Δ3675–3677 deletions, and SΔ241 mutation. Five studies detected the P.1 (Gamma) variant by targeting the N501Y mutation, ORF1a Δ3675–3677 deletions, and the insertion in the 28227–28286 region. In addition, ten studies reported the detection of different lineages of the Omicron (e.g., BA.1 and BA.2) variant by targeting a region of five adjacent SNPs common to BA.1 and BA.2 and distinguished these two variants by detecting the specific 143–145 deletion of BA.1 and the specific LPPA24S (a 9 bp deletion) mutation of BA.2 [100].

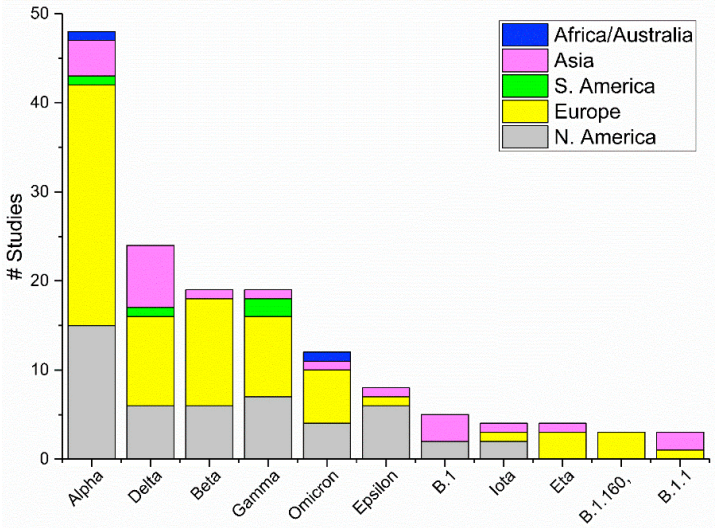

Figure 4. Number of studies targeting or detecting different SARS-CoV-2 variants. Variants are included in the figure only if they are detected by three or more studies.

5. Discussion

The monitoring of SARS-CoV-2 variants in wastewater was established as a promising tool for evaluating spatial and temporal trends of emerging variants at a population level. However, this review showed that the approach was not yet equally popular in resource-limited countries compared to resource-rich ones (Europe and North America). This can be due to a lack of WWS knowledge, awareness, and functional governmental public health institutions in resource-limited countries. Significantly low reporting of the WWS approach in resource-limited settings may indicate a major weakness of current global policy and institutions working on emerging pathogens. It may challenge strengthening the institutions and managing the resources (monetary, technology, and manpower) in resource-limited regions for fighting current and future pandemics. As SARS-CoV-2 demonstrated, the emergence of a communicable disease from one nation can easily sweep through all nations globally.

Indeed, WWS is an economic approach for monitoring emerging pathogens at a population level, and its promotion in developing countries can help fight future emerging pandemics. Many resource-limited countries successfully conducted a variant survey in clinical patients and reported the results in GISAID and WHO reporting systems. However, such studies and reporting are not conducted for wastewater, although the economic cost and analysis technology of one WWS sample analysis is roughly the same as that for the analysis of one single clinical patient sample. Countries with large populations, poor healthcare facilities, and the incapacity of extensive individual clinical testing could greatly upgrade their sentinel surveillance systems by using WWS. The WWS approach provides a near real-time snapshot of the ongoing pandemic [39]. For example, clinical surveillance needs a series of stages for understanding the status of the spread of new variants at a population level: virus colonization to individuals, maturation of symptoms, clinical testing, diagnosis, and reporting. Nonetheless, a variant is excreted to wastewater as soon as it is colonized in individuals and shed from feces, nasal secretion, and other bodily

fluids [21]. WWS is an important complementary survey approach to the conventional clinical or hospital-based epidemiological survey of infectious diseases.

Two approaches, PCR-based (RT-qPCR and RT-dPCR) and next-generation sequencing (NGS) or high-throughput sequencing followed by bioinformatics analysis (Table 2), are widely used for monitoring SARS-CoV-2 variants in wastewater [39]. The PCR-based approaches are relatively fast, simple, sensitive, and cost-effective for monitoring specific variants. These methods target signature mutation regions of the SARS-CoV-2 genome and detect the variant. Targeting the ORF1a gene that exists in many variants and the HV69/70 deletion present in the Alpha variant helps to differentiate this variant from others. For discriminating between variants, European authorities recommend that targeting should cover at least the S gene, particularly in that it encodes the entire N-terminal region and the receptor-binding domain (RBD) corresponding to amino acids [128]. RT-qPCR/RT-dPCR methodologies are often designed as duplex or multiplex, allowing the simultaneous detection of many variants and estimating their percentages among other simultaneously occurring variants. The relatively low cost and low time and labor requirements make these methods (RT-qPCR, RT-dPCR) more suitable for monitoring known variants. The targeting of mutation assays applied to SARS-CoV-2 RNA extracted from wastewater can be a rapid, efficient, and reliable way of monitoring variants introduced and circulated in a community [88]. Compared to RT-qPCR, RT-dPCR was recently popularized and reported as more sensitive than RT-qPCR, as it measures the absolute count of gene copies and is less affected by PCR inhibitors than RT-qPCR [126,129].

Table 2. Comparing pros and cons of sequencing and RT-qPCR/RT-dPCR based methods for detecting SARS-CoV-2 variants.

	Sequencing	**RT-qPCR/RT-dPCR**
Pros	• Can detect all circulating variants, even silently circulating in a population, and enable showing the diversity of circulating variants; • Suitable for early warning of emerging variants; • Accurately provide information about the full mutation patterns specific to all variants; • Possible simultaneous detection of many variants and estimating of their percentages.	• Fast, sensitive, low labor required, and cost-effective for screening particular known variants; • Powerful screening tool; • Simple to use and interpret; • Possible duplexing or multiplexing allows the simultaneous detection of many variants and estimating of their percentages.
Cons	• Relatively expensive, as it demands high reagent and consumable costs, specific equipment, and technical analysis skills; • Labor- and time-consuming as extra bioinformatics knowledge is required; • Multiple lineages in raw sewage complicate the proper assembly of reads to determine the complex sequence circulating in communities.	• Earlier knowledge about the mutated sequence is needed for designing primers and probes; • Detect only the targeted signature mutations and cannot detect non-targeted variants; • Less suitable for tracking new emerging variants and for early warning.

The requirement that PCR-based methods (RT-qPCR/RT-dPCR) use specific primers is a limitation, as these methods cannot detect non-targeted variants. Such primers and probes need to be designed and validated for each new mutant/variant (Table 2). Therefore, PCR-based methods are less suitable for detecting novel variants compared to NGS. For designing a primer, previous information about the region of mutation in the genome is needed. Nevertheless, such information can be obtained for primer design from the GISAID database after clinical testing deposits [130]. Further, a variant-specific reverse transcription-nested PCR approach can be applied to determine the key regions of the viral spike protein.

The next-generation sequencing (NGS)-based approach enables the detection of signature mutations of different variants and provides a real picture of the variant circulation

in real-time [39]. In comparison to the PCR-based method, the NGS-based method can detect all circulating variants (even silently circulating) in a population and enables us to show the diversity of circulating variants. As the NGS-based method covers longer genome coverage and also the design and validation of new assay is not needed for each variant. Additionally, the NGS-based method enables the possibility of retrospective analysis and finding variants circulating earlier in a particular sample. These methods are the most efficient analysis method for monitoring the emergence of the new SARS-CoV-2 variants even earlier than clinical cases. However, NGS-based methods require high reagent and consumable costs, specific equipment, and technical analysis skills. In addition, multiple lineages in raw sewage do not allow the proper assembly of reads to determine the complex sequence circulating in communities [69,93].

Other methods used for monitoring SARS-CoV-2 variants in wastewater were reverse transcription-nested PCR (RT-nPCR) assays followed by Sanger sequencing or NGS analysis, and amplicon sequencing of the only selective gene of SARS-CoV-2 variants instead of targeting the whole or nearly complete genome of SARS-CoV-2 from environmental samples.

WWS played an important role in enabling decision-making among health officials and researchers. It has positive impacts on public health departments and governments to prepare for the possible spread of new variants, though they generally do not pay attention to other variants below the set detectable limits in wastewater due to dilution. The use of WWS also highlights how fast variants of concern transmit from one nation (where the first case is recorded) to others connected via direct or indirect transportation connections, such as land, sea, and air transport routes [26,121]. The detection of Alpha, Beta, Delta, and Omicron variants, amongst others, in multiple countries, is an indication of how global an impact the COVID-19 pandemic had. This makes the use of WWS tools more about understanding and providing adequate control measures in forestalling the spread of SARS-CoV-2. Wastewater surveillance tools can be further developed to track the spread of other infectious diseases [21,22,131–135], especially viruses that can be excreted through human feces and/or urine and saliva. Incorporating WWS as a reliable surveillance tool in a global policy about tackling new and emerging pathogens can be prominent. It may be prominent for strengthening institutions and managing resources (monetary, technology, and human) in resource-limited countries, as the emergence of a communicable disease from one nation can easily sweep through all countries globally, as SARS-CoV-2 is showing.

In addition to its many benefits, WWS of the SARS-CoV-2 variant has some limitations; the most prominent being that it does not reach up to an individual level. Further, wastewater can have many variants in different proportions and PCR inhibitors [126]. Unlike when isolated from an individual case, which consists of a single genome, wastewater samples are likely to contain material from multiple SARS-CoV-2 variants shed from different individuals. Further, each variant at low concentrations and in various stages of genomic integrity because of degradation makes accurate detection challenging [88]. In addition, multiple lineages in raw sewage do not allow for the proper assembly of reads to determine the complex sequence circulating in communities [69,93]. Thus, in the future, more efforts should be put into developing a comprehensive monitoring workflow of SARS-CoV-2 variants, including the sequencing-based screen of unknown variants and the RT-qPCR/RT-dPCR methods-based long-term detection and quantification of specific variants.

Finally, some limitations of this type of systematic review need to be considered while interpreting the findings [29,133]. For example, there is high heterogeneity in the reviewed studies in terms of sample size, target, and detection methods, and in many cases, there can be a lack of transparency in reporting results. For example, there could be reporting biases (most likely positive reports could be more frequently published than negative results [29,133]). Further, there could be target selection bias, particularly while using PCR-based methods, as primers could be targeted only toward some most common variants. Locally circulating variants could guide such interest in communities based on clinical reporting. However, there could already be new variants in communities, which could be detected with WWS.

6. Conclusions

This review revealed that several nations, mostly from resource-rich regions (Europe and North America) actively and regularly engage in WWS to track different variants of SARS-CoV-2. Among different variants, the B1.1.7 (Alpha) variant, which first appeared as an emerging variant during the pandemic, was the most reported (48/80 total studies), followed by B.1.617.2 (Delta), B.1.351 (Beta), P.1 (Gamma), and others in wastewater. All variants reported as WWS followed the same pattern as the clinical reporting of the variants in a timeline and demonstrated that WWS tracked all variants in a timely way when they were developing. Still, developing a highly sensitive, accurate, cost-effective, automated, and reliable tool, which can be practically feasible in both resource-rich and resource-limited settings, for monitoring the trends of current and future COVID-19 outbreaks is highly important for increasing the reliability of WWS.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15061018/s1; Table S1: Summary of literature data on the detection of SARS-CoV-2 variants by wastewater surveillance.

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