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Research article

Global and genetic diversity of SARS-CoV-2 in wastewater

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ABSTRACT

The analysis of SARS-CoV-2 in wastewater has enabled us to better understand the spread and evolution of the virus worldwide. To deepen our understanding of its epidemiological and genomic characteristics, we analyzed 10,147 SARS-CoV-2 sequences from 5 continents and 21 countries that were deposited in the GISAID database up until January 31, 2023. Our results revealed over 100 independent lineages of the virus circulating in water samples from March 2020 to January 2023, including variants of interest and concern. We observed four clearly defined periods of global distribution of these variants over time, with one variant being replaced by another. Interestingly, we found that SARS-CoV-2 water-borne sequences from different countries had a close phylogenetic relationship. Additionally, 40 SARS-CoV-2 water-borne sequences from Europe and the USA did not show any phylogenetic relationship with SARS-CoV-2 human sequences. We also identified a significant number of non-synonymous mutations, some of which were detected in previously reported cryptic lineages. Among the countries analyzed, France and the USA showed the highest degree of sequence diversity, while Austria reported the highest number of genomes (6,296). Our study provides valuable information about the epidemiological and genomic diversity of SARS-CoV-2 in wastewater, which can be employed to support public health initiatives and preparedness.

1. Introduction

The current prevailing consensus posits that SARS-CoV-2 primarily spreads through respiratory droplets, human-to-human contact, fomites, and contact with contaminated surfaces [1]. Nevertheless, the virus has demonstrated an affinity for epithelial cells in the gastrointestinal tract, with the presence of the virus in the feces of COVID-19 patients ranging from 40% to 53% [2–4], indicating the potential for fecal-oral transmission of the virus. Additionally, as fecal matter is disposed into sewer systems and subsequently to wastewater and sewage treatment systems/plants [5], it is plausible to consider sewage as another potential route for transmission of SARS-CoV-2 to humans and animals.

To date, SARS-CoV-2 RNA and/or viral proteins have been detected in water sources of over 58 countries, including wastewater

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treatment plants, medical wastewater, non-potable water, and rivers [6–13]. While there is no direct evidence of infection risk associated with these sources, studies indicate that viable SARS-CoV-2 particles can persist in aerosols for up to 16 h [14] and remain infectious for several days in wastewater [15]. This raises concerns of potential exposure to the virus through contaminated wastewater, particularly in low-income countries with inadequate health and sanitation infrastructure [16,17].

Since the initial discovery of SARS-CoV-2 in wastewater, several independent studies have used genomic and epidemiological surveillance to identify viral variants, track emerging strains, estimate community prevalence, elucidate geospatial distribution of lineages and predict outbreaks in different regions worldwide [13,18,19].

In response to the COVID-19 pandemic, several national and regional Wastewater Surveillance Systems have been established by U. S and European agencies. These organizations include the U.S. National Wastewater Surveillance System (NWSS), the Canadian Water Network (CWN), the Scottish Environment Protection Agency (SEPA) in the United Kingdom, EWAG-2021 in Switzerland, and the Luxembourg Institute of Science and Technology (LIST), among others. These initiatives aim to better understand the spread and community trends of SARS-CoV-2 [20,21], and have contributed to mapping contagion curves, identifying hotspots of activity, evaluating the effectiveness of policies and programs, tracking the spread of new emergent variants, and subsidizing early warning systems for COVID-19 outbreaks. With the help of wastewater surveillance, these organizations have provided valuable insights into the pandemic's progression and have assisted public health agencies in making informed decisions to mitigate the spread of the virus [22].

Despite the wealth of information available, there is currently no comprehensive study with a sufficient number of sequences to analyze the epidemiological and genomic diversity of SARS-CoV-2 in water samples, providing a more holistic understanding of the virus's evolution across different regions over time. Therefore, this study aims to address this gap by evaluating the epidemiology, phylogenetic relationships, and genomic diversity of SARS-CoV-2 sequences from wastewater samples worldwide throughout the first three years of the pandemic. The results of this study will not only enhance our understanding of the virus's potential spread in various geographic areas but also shed light on its behavior before and after public health interventions such as isolation and vaccination.

2. Materials and methods

2.1. Study population

For this study, we obtained 10,147 SARS-CoV-2 sequences from various water sources worldwide through the Global Initiative on Sharing All Influenza Data (GISAID) database [23], including all entries until January 31, 2023. We only included high-quality sequences and excluded those with low coverage and a high number of Ns (>5% Ns). Of the sequences analyzed, 99% were derived from sewage and wastewater sources, while the remaining 1% were from residual wastewater, fresh water, water, bus wastewater and sanitary water. The complete dataset is provided in Table S1.

2.2. Descriptive analysis

The full dataset containing 10,147 SARS-CoV-2 genomes from various water sources was utilized to perform a comprehensive descriptive analysis, taking into account the metadata provided for each genome. The prevalence of variants of interest and concern was examined over time and by country, particularly in regions with high numbers of SARS-CoV-2 water genomes. The graphical representations of the data were generated using R software and the QGIS geographic information system (https://www.qgis.org/es/site/).

2.3. Phylogenetic and mutational analysis

To establish the phylogenetic relationship among the 10,147 SARS-CoV-2 sequences downloaded from the GISAID database, we compared them to 2363 reference genomes from human samples that are representative of SARS-CoV-2 lineages. The reference genomes were downloaded from the Nextclade tool v1.5.4 (https://clades.nextstrain.org/). We assembled a total dataset of 12,510 sequences and aligned them against the reference sequence Wuhan-Hu-1/2019 (MN908947) using MAFFT v7.48 [24]. A maximum-likelihood phylogenetic tree was then reconstructed using IQtree v.2.1.3 [25]. We exported the tree in Newick format and visualized it using iTOL [26], where we made necessary edits to produce a clear representation.

In addition, the dataset was analyzed for Single Nucleotide Polymorphisms (SNPs) and Insertions and Deletions (InDels) using the Nextstrain tool (https://github.com/nextstrain/ncov) and compared to the Wuhan-Hu-1/2019 (MN908947) reference genome. This allowed for a comparison of variants and their prevalence across different countries.

2.4. Statistics analysis

A descriptive analysis was conducted to summarize categorical variables in terms of frequencies and proportions. To explore possible associations between variables, a $\chi 2$ test was implemented. Furthermore, post-hoc tests were performed to make pairwise comparisons between different categories using the *chisq.posthoc.test* function in the R vcd package, with Bonferroni adjustment method. All statistical analyses were conducted using R software (RStudio Team 2019). Two-tailed tests of significance were used, and P-values less than 0.05 were considered statistically significant.

3. Results

3.1. Epidemiology and geographical distribution of SARS-CoV-2 variants from water samples

The global distribution of SARS-CoV-2 variants was analyzed in attempt to understand the genomic variability of the virus throughout the course of the COVID-19 pandemic. The dataset reflected the emergence and prevalence of different variants over time. The analysis revealed four distinct periods marked by the presence of variants of interest/concern (VOI and VOC) (Fig. 1). The first period (December 2020 to January 2021) was characterized by the presence of the Epsilon variant, followed by a second period (February to June 2021) with a predominance of the Alpha variant. The third period (July to December 2021) was dominated by the Delta variant, and a fourth period (January to November 2022) portrayed a transition from Delta to Omicron variants and their subvariants. Omicron was the predominant variant during this period. However, between December 2022 and January 2023, the proportion of Omicron decreased, with a concomitant increase in proportion of other variants (Fig. 1).

The analysis of whole SARS-CoV-2 genomes obtained from water samples revealed a widespread geographical distribution. Europe exhibited the highest number of reported genomes ($n = 6{,}672$, 65.75%), followed by North America ($n = 3{,}419$, 33.69%), Asia ($n = 25$, 0.24%), South America ($n = 24$, 0.23%) and Africa ($n = 7$, 0.07%). Notably, 94% of the genomes from Europe were reported from Austria, while close to 100% of the genomes from North America were reported by the United States (Fig. S1). Distribution of genomes by country revealed that nine countries formed the highest number of genomes, namely Austria ($n = 6296$), United States ($n = 3417$), France ($n = 147$), Italy ($n = 106$), Liechtenstein ($n = 61$), Germany ($n = 41$), India ($n = 16$), Brazil ($n = 16$) and Ukraine ($n = 11$). In all cases, an elevated representation of variants was observed with a clear predominance of VOI/VOC (Alpha, Delta and Omicron variants) (Fig. 2).

The United States stands out for displaying a high diversity of SARS-CoV-2 genomes, as evidenced by the detection of Epsilon, Alpha, Beta, Delta, Gamma, Mu, Omicron, and other lineages of less concern in water samples (Fig. 2). This pattern can be explained by the ongoing genomic surveillance efforts carried out in the US, which have resulted in the procurement and analysis of 3417 genomes obtained from water samples. As for other countries, the dynamics of the virus and the emergence of VOI/VOC have led to changes in the predominance of these variants over time. Specifically, through initial reports of Alpha variants, followed by a period of Delta predominance, and a final, prolonged period characterized by a higher and sustained prevalence of Omicron and its subvariants (Fig. 2, Fig. S2). Brazil had also a report on the Gamma variant (Fig. 2); this likely due to the emergence of this variant in the country. Furthermore, a statistically significant association between variants and continents was observed (Chi-squared = 3351.5, df = 85, p-value <2.2e-16).

3.2. Phylogenetic relationships of SARS-CoV-2 sequences

Phylogenetic analysis of 12,510 SARS-CoV-2 genomes, including 10,147 sequences from water samples and 2363 reference genomes from human samples (representative of SARS-CoV-2 lineages), revealed the presence of five distinct subpopulations, which were clustered into well-supported nodes (Fig. 3). Cluster-1 (C1) was the most diverse and included 5750 genomes consisting of 2316 reference genomes and 3434 genomes from water sequences analyzed in this study. Cluster-2 (C2) was composed exclusively of 40 water sequences, with 18 from North America, 21 from Europe and one from Asia. Cluster-3 (C3) included 2155 genomes with 30 reference genomes and 2125 water samples distributed as follows: 1398 from North America, 703 from Europe, 16 from South and

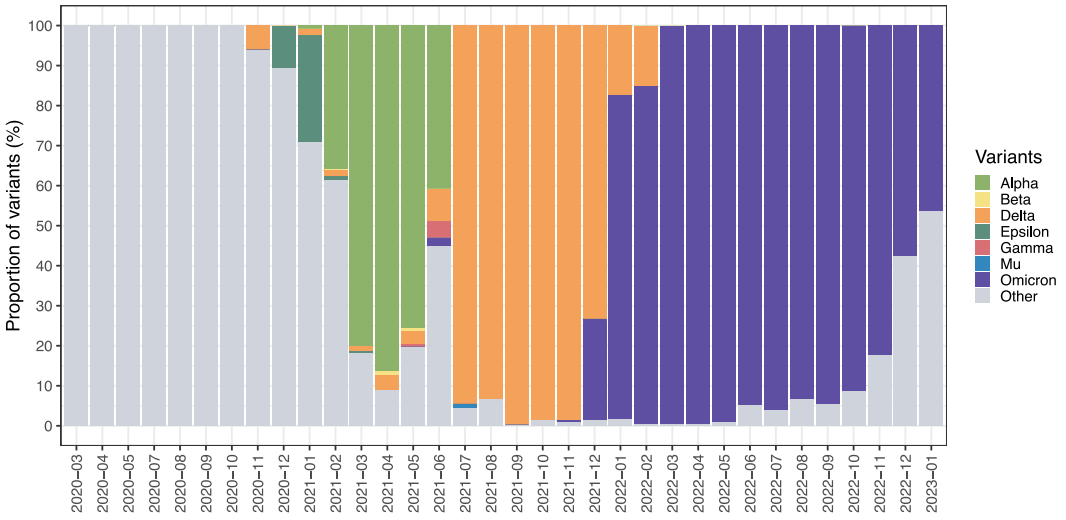

Fig. 1. Proportion of SARS-CoV-2 variants of interest/concern from water samples. Representation of the proportion of variants through the time of 10,147 publicly available genomes from water samples.

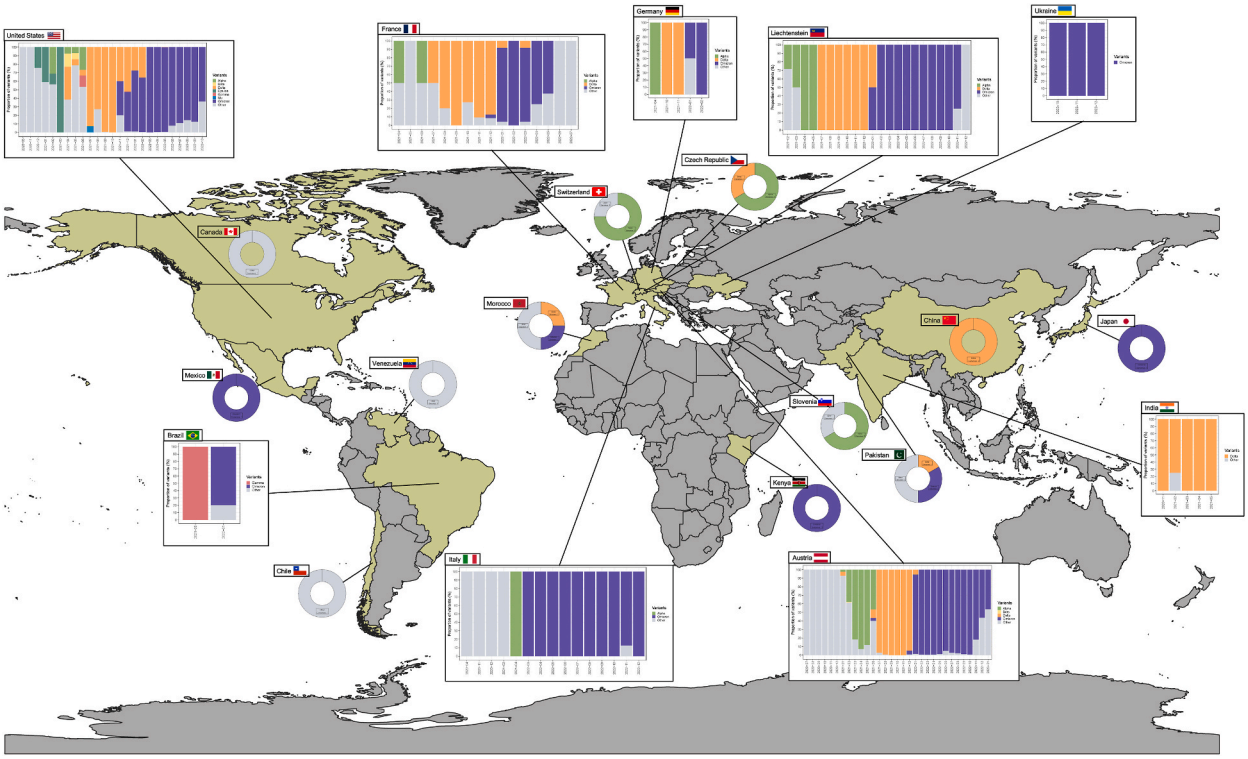

Fig. 2. Geographical origin of the publicly available SARS-CoV-2 genomes. Representation of the variants' proportion of the public SARS-CoV-2 genomes from water samples discriminating by the country origin. Bar plots of the variants over the time of the countries with the highest report of genomes (>11) and donut chart with the proportion of variants and the number of genomes respectively for the remain countries.

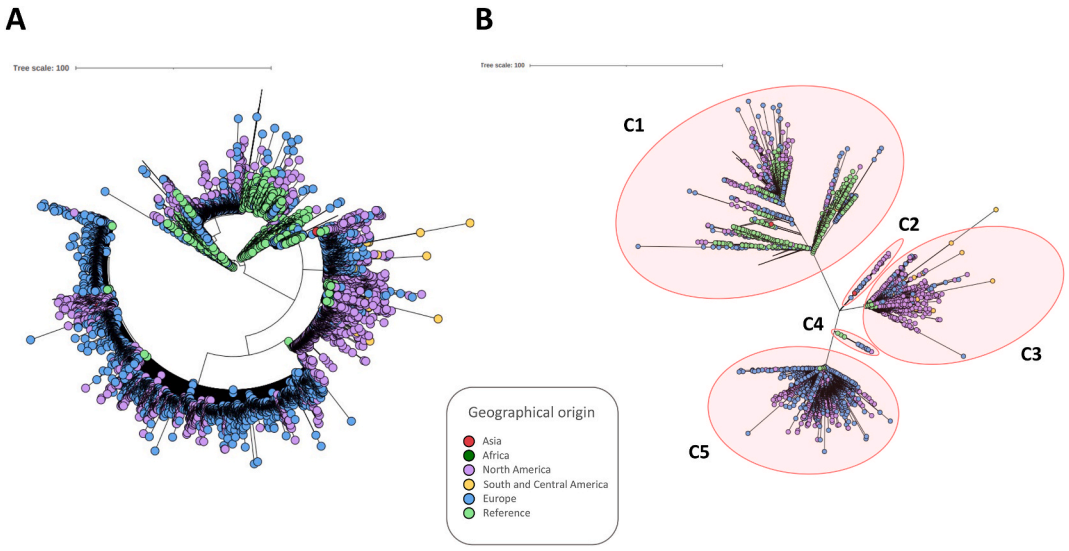

Fig. 3. Phylogenetic analysis of SARS-CoV-2 from water samples in the global context. A. Phylogenetic relationship between the 12,510 sequences download of GISAID database. The tree was construct using 10,147 sequences from water samples and 2363 sequences of reference genomes. **B.** Unrooted maximum-likelihood phylogeny, the red circles represent each of the cluster identified. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Central America, 4 from Asia and 4 from Africa. Cluster-4 (C4) comprised 17 sequences, consisting of 4 reference genomes and 13 sequences from water samples, 11 from Europe and 2 from the USA. Finally, cluster-5 (C5) included 4548 genomes with 13 reference genomes and 4535 from water samples, 3494 from Europe, 1040 from North America and one from Asia (Fig. 3).

3.3. Mutational profile of SARS-CoV-2 lineages from water samples

The analysis of diversity in the 10,147 sequences examined in this study, identified 93 amino acid substitutions that occurred in more than 10% of the genomes analyzed. Among these, two substitutions (P314L and D614G) were consistently present in over 98% of the water samples studied, while 28 mutations were detected in more than 50% of sequences. The majority of these substitutions were located in the spike protein, with other located in the ORFs 1 ab, M and N.

In order to focus on mutational analysis of variants of interest and concern (VOI/VOC), we selected 9656 sequences out of the 10,147 included in this study. These sequences belonged to six different variants, with 6713 belonging to Omicron, 2261 to Delta, 632 to Alpha, 43 to Epsilon, 4 to Gamma, and 3 to Beta. We compared each sequence to the reference strain Wuhan-Hu-1/2019 (MN908947) and found that Omicron had the highest number of non-synonymous substitutions, with 71 in total, 58% of which were located in the ORF S. Delta and Gamma variants followed with 45 and 27 substitutions, respectively. The Beta variant had the lowest number of substitutions with only 19 (Fig. 4). Moreover, the results showed that the Omicron and Alpha variants had the highest number of insertions and deletions (InDels), predominantly in the ORF S (Fig. 4).

The non-synonymous substitutions identified in each variant were compared among different countries. For Omicron variant, the country with the highest number of polymorphic sites was USA, with 72 out of 2459 sequences analyzed. France and Austria followed with 71 and 70 polymorphic sites in 43 and 4405 sequences, respectively. Germany had the lowest number of substitutions with only 43 in 4 sequences analyzed. In the case of the Alpha variant, France and the USA had the highest number of substitutions, with 65 (in 4 sequences) and 66 (in 13 sequences), respectively. Interestingly, despite having a high number of available sequences (587), Austria had the lowest number of substitutions for the Alpha variant with only 21. For the Delta variant, France and India showed the highest number of polymorphic sites with 73 (in 53 sequences) and 71 (in 15 sequences), respectively. Austria had the lowest number of polymorphic sites for the Delta variant with only 33 in 1392 sequences analyzed.

In order to gain further insight into the temporal dynamics of Omicron, which is currently the most prevalent variant worldwide, we performed a mutational analysis over time (Fig. S3). Our analysis revealed that some SNPs disappeared, and others were introduced and maintained over time in genomes deposited from several countries including the US, Austria, Liechtenstein, Italy, and France (Fig. S3). Additionally, we identified several non-synonymous substitutions not previously reported for each variant in the outbreak. info database (https://outbreak.info/). Among the additional polymorphic sites, Omicron displayed the highest number of substitutions (31), with the majority of them located in the ORFs 1a and S (22% and 51%, respectively). Epsilon variant showed 12 substitutions, of which 58% were in the ORF1a region. Alpha variant had the lowest number of additional polymorphic sites, revealing only one non-synonymous substitution (Table S2).

4. Discussion

The use of wastewater-based epidemiology and genomic surveillance has emerged as a highly effective tool for monitoring the spread of SARS-CoV-2 in the community. By analyzing wastewater samples, researchers have been able to estimate the prevalence of the virus, identify its geographical distribution, and track its genetic diversity [13,27]. This monitoring has been crucial not only for

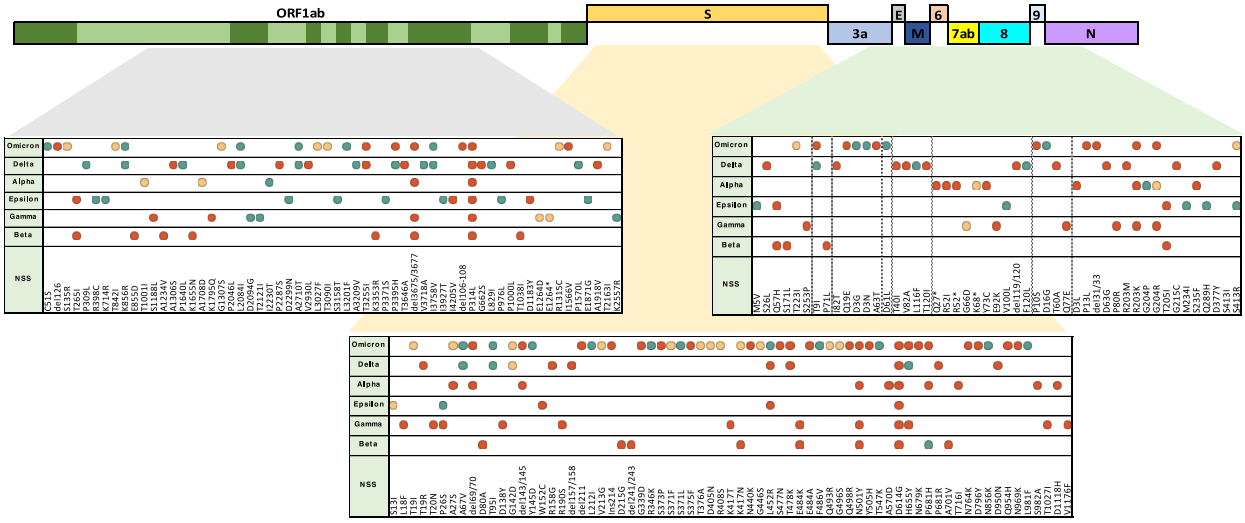

Fig. 4. Nucleotide diversity analysis of genomes from water samples. Analysis of nucleotide diversity by comparing the 10,147 SARS-CoV-2 sequences and the Wuhan reference sequence (Wuhan-Hu-1/2019 (MN908947)). Each panel represent the non-synonymous substitutions (NSS) found in the VOC/VOI variants through of the different ORF. The green circles represent the mutations found between 10 and 30% of genomes analyzed, the orange circle those identified between 30 and 70% and the red circles those found in more than 70% of the genomes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

understanding the course of the COVID-19 pandemic, but also for detecting cryptic lineages that resist neutralizing antibodies [28] and issuing early warnings of potential outbreaks.

Previous studies have reported the detection of certain variants of epidemiological concern, such as the Alpha and Delta variants, in wastewater before they were observed in clinical samples [29–31]. This highlights the importance of wastewater-based epidemiology and genomic surveillance in monitoring the spread of the virus and detecting emerging variants early on. By providing a more comprehensive view of the epidemiological and genomic behavior of SARS-CoV-2 from water samples reported across various countries throughout the first three years of the pandemic, our study contributes to the growing body of knowledge on the evolution of the virus and the development of effective strategies for controlling its spread.

An extensive analysis of 10,147 SARS-CoV-2 genomes obtained from water samples collected between March 2020 and January 2023 has revealed a remarkable diversity of the virus. This investigation uncovered more than 100 independent lineages of the virus, including five variants of concern (Alpha, Delta, Gamma, Beta, and Omicron) and two variants of interest (Mu and Epsilon) (Fig. 1). Through phylogenetic analysis (Fig. 3), the study has also highlighted the mutational dynamics that the virus has undergone since the beginning of the pandemic. These findings raise significant concerns about the potential risks associated with the circulation of variants that exhibit high degrees of virulence, transmissibility, and immune escape ability [25,32–36]. Such variants could not only pose a risk of contagion to humans and animals, potentially resulting in the emergence of new outbreaks, but also the expansion in the number of SARS-CoV-2 hosts and the possible development of novel mutations. In this context, the study highlights the high genetic heterogeneity of SARS-CoV-2 in water samples, with the emergence of new variants of concern and interest and reinforces the need for sustained surveillance of the virus in wastewater. Such measures are critical in reducing the spread of the virus and in informing public health responses to potential new outbreaks.

Globally, a clear pattern of one variant rapidly replacing another over time was observed (Fig. 1), which was consistent with the profile of variants observed from samples from active cases during the pandemic [12,37,38]. In the majority of analyzed countries, the Omicron variant swiftly supplanted the SARS-CoV-2 Delta variant, spreading rapidly and becoming the predominant strain within the initial weeks. This prevalence was sustained for slightly over a year, as depicted in Fig. 2. These findings align with the high degree of transmissibility, infectivity, and short incubation period demonstrated by the Omicron variant over the Delta variant [39,40], which could cause prolonged infectiousness with unapparent symptoms and consequently, rapid viral spread. Additionally, the robust replication fitness of the Omicron variant, which has favored the generation of a high number of lineages, some of which have been detected in wastewater [41], underscores the need for continued implementation of coordinated workflows between wastewater-based genomic and epidemiological surveillance. This will allow for the rapid detection, tracking, and analysis of the spread dynamics of new SARS-CoV-2 variants.

Despite some limitations - such as low sampling rates in certain countries (e.g., Brazil, India, Ukraine), gaps in data during specific periods (e.g., Italy had no available sequences from May 2021 to February 2022), and a lack of information in some countries - the available data confirmed a correlation between the viral load of SARS-CoV-2 detected in wastewater and clinically confirmed cases during the analysis period. These findings highlight the usefulness of wastewater-based epidemiological and genetic surveillance, not only for identifying circulating viral lineages, but also for accurately tracking the viral strain in an epidemic context. Despite the limitations, the results of this study underscore the importance of continuing to monitor and analyze wastewater for the presence of SARS-CoV-2 in order to better understand its circulation and evolution.

After analyzing the complete dataset, it became clear that Austria had reported the highest number of SARS-CoV-2 sequences ($n = 6296$) compared to other countries (Fig. S1). The availability of robust datasets in Austria allowed the country to (i) detect emergent variants before clinical cases were officially confirmed [42] (ii) establish national wastewater monitoring programs [43] and (iii) deduce public health-relevant epidemiological indicators such as relative abundance and reproduction numbers for SARS-CoV-2 variants [44]. These efforts have contributed significantly to generating early warning systems, evaluating the effectiveness of measures, and facilitating decision-making processes by public health authorities. Such comprehensive approaches provide valuable insights into the viral dynamics, the efficacy of interventions, and the surveillance of new viral variants, ultimately contributing to better public health outcomes.

The observation of a high number of SARS-CoV-2 genomes in Austrian wastewater highlights the country's significant investment in understanding the epidemiology, diversity, and monitoring of wastewater-based SARS-CoV-2. However, this also brings to light the challenges and low interest in identifying and sequencing SARS-CoV-2 from environmental sources, especially in developing countries. To date, different studies have described that the low viral concentration, the presence of inhibitory substances or the use of chemical or physical treatments, could cause viral inactivation and modifying viral genome and proteins [45]. These limitations emphasize the need to prioritize the development of new and better strategies to recover, sequence, and analyze the SARS-CoV-2 genome from environmental samples. Furthermore, there is an urgent need to invest in low- and middle-income countries to improve their operational capacity and enhance their epidemiological surveillance tool.

The phylogenetic analysis of the SARS-CoV-2 sequences from water samples in this study revealed a close relationship between many of them and human sequences (Fig. 3), consistent with the findings of Islam et al., [46]. However, 40 sequences, including 18 from the USA, 21 from Austria, and one from Pakistan, did not show this phylogenetic relationship (Cluster-2) (Fig. 3).

The presence of mutations, including N501Y, S477 N, E484A, and Y505H, previously identified in cryptic lineages by Schumann et al. [47] and Smyth et al. [12,12,47], along with their detection in some sequences analyzed here (mainly from the USA and Europe) (as shown in Fig. 4), suggests that cryptic lineages of SARS-CoV-2 may be circulating in other geographic areas. Additionally, the displacement of the Omicron variant by other lineages, referred herein to as "others" (Figs. 1 and 2), raises the possibility of the existence of cryptic lineages that have not been detected in clinical surveillance but are mainly identified in wastewater from the United States [12,28].

This is especially concerning as these lineages may expand the virus's tropism and evade neutralization by monoclonal antibodies [48], potentially leading to a new health emergency. Several hypotheses can explain these findings. One such hypothesis is the undetected spread of these lineages within the human population from nasopharyngeal samples. Some SARS-CoV-2 variants replicate exclusively in the gastrointestinal tract and may only be detectable in fecal samples [49]. Another possibility is the spread of lineages from non-human sources, such as cats, rats, and dogs, and/or other abiotic niches, as previously described [12,28,50].

While there is currently insufficient evidence to suggest that SARS-CoV-2 can be transmitted through sewerage systems, with or without wastewater treatment, it is crucial to implement early detection and continuous monitoring of this variant through water samples to prevent significant expansion. Conducting deeper sampling can also reveal the true extent of these findings.

From a biological perspective, we have limited knowledge about the respective roles of biotic and abiotic factors in shaping the evolutionary path of SARS-CoV-2 across various water environments. Moreover, there is a need to investigate how these multidimensional environmental factors interact to influence viral populations of SARS-CoV-2. This intriguing evolutionary question undoubtedly warrants further exploration following an in-depth metapopulation dynamics approach. SARS-CoV-2 may utilize water sources in a way similar to other human viral pathogens like enteroviruses [51,52] to enhance its spatial conditions. By exploiting the water niche, the virus could potentially spread on a large scale, affecting its evolutionary ecology through environmental response and genetic changes resulting from high mutation and recombination events, much like other coronaviruses [53]. Hence, further studies are needed to understand the ever-changing adaptive landscape of SARS-CoV-2 variants, including their diversity in response to environmental sources such as water, and the potential evolutionary outcomes.

Another interesting finding was the close phylogenetic relationship observed between SARS-CoV-2 water from different geographical regions, such as the observed by Islam et al. [46]. In this study, we identified phylogenetic relationship among SARS-CoV-2 water samples from South America, Europe, and the USA (Fig. 3). Recent findings have highlighted the crucial role of human movements in the worldwide spread of SARS-CoV-2 variants, as documented throughout the COVID-19 pandemic [46,54,55]. These findings highlight the crucial role of human movements in the spread of SARS-CoV-2 variants worldwide, as has been documented throughout the COVID-19 pandemic [46,54,55] and suggest that the relaxation of measures such as the elimination of negative PCR test as a prerequisite for travel, the reception of travelers from highly endemic areas, and the elimination of mask in indoor areas or on public transport, all as a consequence of vaccination and the decrease in the number of positive cases, may contribute to the transmission and spread of the virus, particularly among pre-symptomatic or asymptomatic population [56].

It is worth noting that 98% of the water sequences analyzed in this study revealed the presence of two amino acid substitutions: P314L (ORF1b) and D614G (ORF S) (Fig. 4), which are strongly associated with viral entry and replication [57]. These mutations are also commonly found in clinical human samples [58] and non-human sources [50]. More than 60% of the analyzed sequences had mutations associated with viral replication (ORF1ab:T32551), increase infectivity and transmission (N:G204R, N:R203K, S:T478K, S:H665Y) [59,60] and immune evasion (S:P681H) (Fig. 4). Such findings suggest that different variants of SARS-CoV-2 are present in wastewater and could potentially affect disease severity, diagnosis, and the effectiveness of treatment and/or vaccines. Therefore, continuous monitoring and tracking of SARS-CoV-2 variants from wastewater should become a priority for local authorities in order to prevent a new public health problem. As such, it is critical that local authorities prioritize continuous monitoring and tracking of SARS-CoV-2 variants from wastewater to prevent the potential emergence of a new public health crisis.

Additionally, we conducted an investigation to assess the number of substitutions in the Omicron, Alpha, and Delta variants and compared them among several countries. Beta, Gamma, Epsilon and Mu variants were not included in the analysis because the available sequences came from just one country. Our results indicated that the water samples from France and the USA presented the highest degree of sequence diversity in three of the variants evaluated, in comparison to Austria, which reported the highest number of genomes. These findings suggest that the observed differences in sequence diversity could be associated with factors such as the number of inhabitants in each country, (USA: ~335 mill, France 65 mill and Austria 9,1 mill inhabitants) https://www.census.gov/popclock/, https://www.worldometers.info/world-population/france-population/, https://www.worldometers.info/world-population/austria-population/, the number of COVID-19-positive cases and the number of active-cases https://www.trt.net.tr/espanol/covid19.

Our findings highlight the relationship between the circulation of SARS-CoV-2 in the human population and its presence in wastewater, as previously reported [61]. Furthermore, our results suggest that the relaxation of public health and social measures may increase the transmissibility of SARS-CoV-2, leading to a higher likelihood of the emergence of new mutations (Fig. S3) that could affect the efficacy of diagnosis and prevention programs. Our study underscores the need for continuous monitoring and surveillance of SARS-CoV-2 variants in wastewater, which could be a valuable tool for predicting and managing future outbreaks.

Several hypotheses have been proposed to explain why cryptic SARS-CoV-2 lineages have been found in wastewater samples but not in clinical samples. One of these hypotheses is associated with undetected COVID-19 infections that lead to a decrease in testing, sequencing, and overall detection of the virus. Another possibility is non-reporting of cryptic lineages in public databases due to their low representation in clinical samples. Additionally, the presence of these lineages in physically distinct locations of the nasopharyngeal area can make them undetectable by routine clinical tests. Finally, it is possible that these lineages are of non-human origin [12,28].

Overall, this study presents some important findings, but it also has several limitations that need to be acknowledged. Firstly, the number of genomes available in some countries is limited, which restricts the ability to track variants over time and to analyze the potential effect of vaccination on viral spread. Therefore, larger, and more diverse datasets are required to provide a more comprehensive understanding in the landscape of viral diversity circulating in wastewater. Secondly, it is essential to expand the genomic surveillance of wastewater through different geographical areas as well as to include non-human sources. This approach could help predict SARS-CoV-2 hotspots and identify the origin of novel cryptic lineages that may pose a public health threat in the future. Lastly,

it is crucial to investigate the potential role of wastewater in SARS-CoV-2 transmission. By addressing these limitations, we can better understand the dynamics of SARS-CoV-2 circulation and take proactive measures to mitigate its spread.

5. Conclusions

The presence of numerous SARS-CoV-2 lineages in water samples and the potential circulation of cryptic lineages in various geographic locations highlights the need to develop effective treatment strategies to eliminate or reduce SARS-CoV-2 from wastewater. While SARS-CoV-2 transmission through water sources remains yet to be confirmed, its proven presence in different water sources may pose a latent threat to aquatic and wildlife animals. Additionally, this study emphasizes the importance of epidemiological and genomic surveillance as an early warning system for SARS-CoV-2 detection and spread. To this end, we suggest expanding epidemiological and genomic surveillance to other water sources such as river water, wastewater produced by hospitals, industries, households, etc., as this would help predict critical and sensitive points of contamination by SARS-CoV-2 and improve intervention programs.

Data availability statement

Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available alongside your article upon publication.

Has data associated with your study been deposited into a publicly available repository?Yes.

Please provide the name of the repository and the accession number here.

Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available alongside your article upon publication.

Has data associated with your study been deposited into a publicly available repository?

"Data is available on the corresponding supplementary files.

Question	Response
Data Availability Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available alongside your article upon publication. Has data associated with your study been deposited into a publicly available repository?	Yes
Please provide the name of the repository and the accession number here. as follow-up to "**Data Availability** Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available alongside your article upon publication. Has data associated with your study been deposited into a publicly available repository? "	Data is available on the corresponding supplementary files

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CRediT authorship contribution statement

Luz Helena Patiño: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nathalia Ballesteros:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation. **Marina Muñoz:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Angie Lorena Ramírez:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation. **Sergio Castañeda:** Writing – review & editing, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Luis Alejandro Galeano:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization. **Arsenio Hidalgo:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization. **Alberto Paniz-Mondolfi:** Writing – review & editing, Visualization, Validation, Supervision, Resources. **Juan David Ramírez:**

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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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Appendix A. Supplementary data

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