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Omicron and Delta variant prevalence detection and identification during the fourth COVID-19 wave in Mexico using wastewater-based epidemiology

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ABSTRACT

Objectives: To identify the SARS-CoV-2 variants Delta and Omicron during the fourth wave of the COVID-19 pandemic in Mexico using samples taken from 19 locations in 18 out of the 32 states.
Methods: The genetic material concentration was done with PEG/NaCl precipitation, SARS-CoV-2 presence was confirmed by reverse transcriptase-quantitative polymerase chain reaction assay, the variant detection was carried out using a commercial mutation detection panel kit, and variant/mutation confirmation was done by amplicon sequencing of receptor-binding domain target region. The study used 41 samples.
Results: The Delta variant was confirmed in two samples during August 2021 (Querétaro and CDMX) and in three samples during November 2021 (Aguascalientes, Ciudad Juárez campuses, and Nuevo Leon). In December 2021, another sample with the Delta variant was confirmed in Nuevo Leon. Between January to March 2022 only the presence of Omicron was confirmed, (variant BA.1). Additionally, in this period six samples were identified with the status "Variant Not Determined".
Conclusion: To our knowledge, this study is one of the first to identify Omicron and Delta variants with polymerase chain reaction in Mexico and Latin America and its distribution across the country with 56% Mexican states making it a viable alternative for variant detection without conducting a large quantity of sequencing of clinical tests.

Introduction

Since the first reports of novel pneumonia cases in Wuhan, China; COVID-19 has caused a pandemic unprecedented in recent history mainly fueled by global mobility [1]. The SARS-CoV-2 ribonucleic acid (RNA) genome size is ~ 29.9 kb and shares sequence homology with SARS-COV at ~ 78% and Middle East Respiratory Syndrome coronavirus (MERS-CoV) at ~ 50% and includes two open reading frames (ORFs) that correspond two-thirds from full genome shown in Figure 1A as gene domains 1a and 1b, leading the translation into pp1a and pp1b polypeptides that are cleft by two cysteine proteases (papain-like protease [PLpro], or nsp3, and a 3C-like protease [3CLpro] or nsp5) encoded in the virus genome which results in 16 nonstructural proteins [2]. The rest of the genome encodes for structural proteins such as spike protein monomer (S) consisting of receptor-binding domain (RBD) subunit (S1) and transmembrane subunit domain (S2), and nucleocapsid protein (N), membrane protein (M) and envelope protein (E) which facilitate virus-like particle formation (Figure 1B) [3]. The S proteins on the virus envelope are where the S1 protein/receptor triggers the mechanism for SARS-CoV-2 to infect its host (Figure 1C) by direct fusion with the viral envelope and host membrane or by fusion within the endosome after endocytosis [4]. The infection mechanism is through the metallo carboxyl peptidase angiotensin receptor 2 (ACE2) which consists of a transmembrane anchor and an extracellular domain in its cellular membrane form, which is the principal infection form, due to the second form being soluble and circulates in low concentrations. Due to ACE2 being widely expressed in the cells of the lung, liver, intestine heart, kidney, testis, and vascular endothelial cells, SARS-CoV-2 can infect those tissues and lead to brain inflammation and intestinal symptoms [5].

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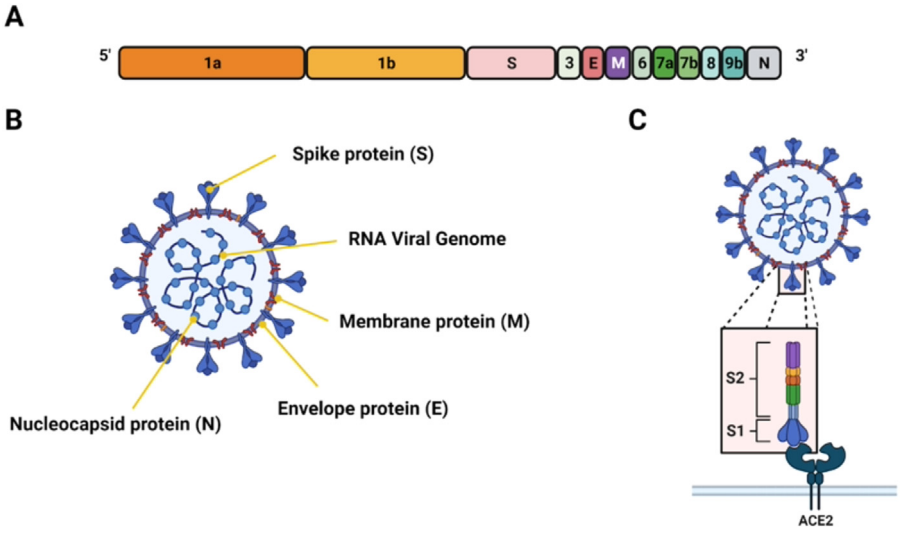

Figure 1. SARS-CoV-2 architecture genome (a); SARS-CoV-2 viral particle representation (b); and, structure and infection mechanism by ACE2 recognition (c).

As the virus spread progressed so did the multiple strategies whose common goal is to minimize the impact on our society. One of them focuses on the surveillance of wastewater, which is one of the main strategies employed to track present and future variants. This type of surveillance supposes the advantage of detecting its presence even in asymptomatic populations, by doing wastewater-based epidemiology (WBE), new variants can be detected before confirming their presence in a single region. That was the case when the N3 gene was detected 6 days earlier than the first case of COVID-19 reported in the Netherlands by using this powerful strategy [6]. Once variants are detected, it is important to proceed with their identification.

SARS-CoV-2 mutates giving rise to different variants, such mutations affect its transmissibility, disease severity, and its response to neutralizing antibodies and drugs [7]. For example, modifications in the RBD of the S protein influence its affinity to ACE2 consequently altering viral entry into the human body [7]. The importance of being able to differentiate between variants, and take measures, accordingly, generates a positive impact on the public health emergence of these variants. World Health Organization has created two categories: variants of interest (VOI) and variants of concern (VOC) [8].

The Delta variant was first recognized in India in the first months of 2021 [9]. In South Africa, it originally caused 1020 daily cases and the number grew by more than 20% weekly, replacing the Beta variant as the most widespread VOC in the region [10]. The characteristic of this variant is its enhanced transmissibility associated with its increased infectiousness, with higher viral loads in its host than other variants. Although its epidemiology varies according to the population's characteristics and access to testing [11]. Moreover, the Delta variant has a short incubation period, transmission is 67% higher compared to the Alpha variant and patients with COVID-19 have a higher risk of hospitalization and mortality [11]. It also presents a reduced amplification of the RNA-dependent-RNA-polymerase (RdRp) gene target, which can be used as one of the main markers to detect the variant [10].

The B.1.1.529 VOC is known as Omicron, originally detected in the United Kingdom on November 26, 2021 [9]. Omicron exhibits multiple mutations in the RBD of the S protein and N-terminal domain region, which allows it to enter the cell more efficiently and cause greater infectivity. It has a transmission rate of 3.19 times higher compared to that of the Delta variant [12]. The present COVID-19 vaccines are less effective in preventing infection with Omicron, and the variant has caused many breakouts even among vaccinated individuals [12]. Omicron's infectivity is estimated to be around 10 times the original variant, and twice the Delta variant [13]. Due to a deletion in amino acids 69 and 70 that codes for the S protein, Alpha and Omicron variants were not detected by commercial diagnostic assays such as Thermo Fisher TaqPath (Waltham, USA) [10].

Omicron and descendant lineages (Subomicron)

The first Omicron sublineage that appeared was BA.1, but later BA.2 was detected on November 17, 2021, in South Africa and since then it has gained concern as its circulation, compared with the BA.1, has increased globally [14]. BA.2 differs from BA.1 in its genetic sequence, which changes the structure of the S and other proteins, which results in a more transmissible virus. However, the data available is lacking in terms of the severity that this lineage can cause compared to BA.1 [15].

BA.1 (B.1.1.529.1)

In April 2022, variant B.1.1.529.1 (the original Omicron variant of SARS-CoV-2) accounted for more than 9 out of 10 Omicron variant cases globally. Thus far, this variant has spread to more than 130 countries and is characterized by a significant number of mutations. At least 37 mutations have been reported in the S protein alone [16]. According to the World Health Organization, the chances of infection and replication in the upper respiratory tract (nasal cavity, nasopharynx, and larynx) are higher for the variant BA.1, while the Delta infection and replication process takes place in the lower respiratory tract (lungs and bronchioles). Due to this difference, the speed in the transmission mechanism present in the BA.1 variant could be explained. Current COVID-19 vaccines appear to provide strong protection against serious illness and death from BA.1 infection. In addition, the administration of a third "booster" dose was reported to provide greater protection [17].

According to clinical testing and sequencing in Mexico, the third wave was triggered by the Delta variant [18], while the increased cases during the fourth wave were triggered by the Omicron variant [19]. In this study, we aim to identify the prevalence of the two main variants of SARS-CoV-2 reported to be prevalent during the fourth wave in Mexico. Additionally, to analyze when the Omicron variant started to be detected through WBE. Moreover, this study evaluates if it is possible to detect and identify the variants of SARS-CoV-2 in wastewater samples using a commercial mutation panel assay and determine the prevalence of the Delta and Omicron variants throughout the country. In order to observe the spread and epidemic evolution of SARS-CoV-2 variants in Mexico. Finally, to evaluate the challenges and opportunities in fast and accessible variant detection by polymerase chain reaction (PCR).

Figure 2. Wastewater sample collection points in all campuses of Tecnologico de Monterrey in Mexico.

Material and methods

Wastewater sample collection, concentration, and detection

Wastewater samples were taken from the WWTPs and buildings of Tecnológico de Monterrey campuses across Mexico (Figure 2) during the fourth COVID-19 wave reported in Mexico, from November 11 to March 25, 2022, grab samples were collected weekly, following the procedures outlined in Norma Mexicana PROY-NMX-AA-003-SCFI-2019 [20]. Tecnológico de Monterrey is an education institution with a nationwide presence, the institution is present in 19 of 32 federal identities (60% of the federal identities in Mexico), also the student community is characterized by being from a large number of municipalities in each state, having a representative number of people that cover the territory of each state in which Tecnológico de Monterrey is present. During the present study samples were collected and stored at 4˚C before being processed. Upon arrival, a total of 70 ml aliquots per sample were concentrated using PEG/NaCl precipitation method [21]. RNA was extracted following Water DNA/RNA Magnetic Bead Kit (IDEXX, Westbrook, ME) manufacturer's protocol. SARS-CoV-2 quantification was performed with reverse transcriptase-quantitative PCR (RT-qPCR) assays, and SARS-CoV-2 RT-PCR Test (IDEXX, Westbrook, ME) was employed. In addition, Applied Biosystems QuantStudioTM 3 Real-Time PCR System with the QuantStudio Design and Analysis Software 1.3 (Thermo Scientific, Waltham, MA) was used.

Mutation detection panel

A total of 34 RNA samples with cycle threshold (CT) <30 for SARS-CoV-2 N1/N2 gene detection were analyzed using TaqMan™ SARS-CoV-2 mutation panel (Thermo Scientific), using the Applied Biosystems QuantStudioTM 5 Real-Time PCR System with the QuantStudio Design and Analysis Software 1.3 (Thermo Scientific) and the genotyping analysis module was used. Only samples where SARS-CoV-2 was detected in high viral loads were considered (CT <30), according to the manufacturer's instructions for this panel.

A total of five reactions per sample were performed to determine the genotype of the SARs-CoV-2 circulating lineages. The assessed mutations in each of these five reactions were Q493R, L452R, E484Q, P681R, and T478K. Whereas for Delta the reactions are wild type (WT), mutant (MUT), WT, MUT, and MUT, and for Omicron they are MUT, WT, not available genotype (NA), NA, and MUT, respectively. WT indicates the presence of the reference allele in the genomes present in the selected samples. MUT indicates the presence of the alternative allele or mutation in the genomes contained in the selected samples. NA (not available genotype) suggests that any probe (WT nor MUT) did not hybridize on the targeted nucleotide change because of the presence of more than two alleles in that position, meaning that this position is a multiple nucleotide polymorphism (MNP) and additional probes targeting these changes would be needed. These probes were not included in this study.

All of the mutations are located in the S gene. A combination of these mutations contributed to discriminating between Delta and Omicron SARS-CoV-2 lineages. The specific genotype at the five assessed genomic positions using the probes contained in the TaqMan™ SARS-CoV-2 Mutation Panel.

Mutation confirmation by amplicon sequencing

The undetermined samples and omicron-positive samples (14) were sequenced to determine the sublineages of the SARS-CoV-2 Omicron variants. Amplicon sequencing of the RBD was performed using the methodology reported by Smyth et al. [22]. This RBD targeted region spanned S protein amino acid residues from 412 to 579. Some of the defining mutations specific to Omicron and its sublineages are located within this subregion of the RBD. The following table shows the relevant mutations for Delta and Omicron lineage discrimination that can be identified using this approach (Table 1).

Briefly, 5 µl of RNA extracted from wastewater samples were used for the RT-PCR, using the loci-specific primers to amplify the RBD region using the Superscript IV One-Step RT-PCR System (Thermo Fisher, Waltham, MA, USA) followed by a secondary PCR using 5 µl of the resulting amplicon from the previous RT-PCR as template, with gene-specific primers containing 5′ adapter sequences. And a third PCR to add adapter sequences is required for Illumina cluster generation with the forward and reverse primers previously used by Smyth et al. [22]. A final amplicon library pool was created using the amplified products from each PCR reaction mixed to create a single pool. The amplicon pool was

Table 1
Relevant mutations for the Delta and Omicron lineage discrimination.

BA.1.617.2 (Delta)[a]	BA.1	BA.2	BA.4	BA.5	BA.2.12.1
..	S:K417N	S:K417N	S:K417N	S:K417N	S:K417N
..	S:N440K	S:N440K	S:N440K	S:N440K	S:N440K
S:L452R	..	..	S:L452R	S:L452R	S:L452Q
..	S:G446S	..	..	..	..
..	S:S477N	S:S477N	S:S477N	S:S477N	S:S477N
S:T478K	S:T478K	S:T478K	S:T478K	S:T478K	S:T478K
..	S:E484A	S:E484A	S:E484A	S:E484A	S:E484A
..	..	..	S:F486V	S:F486V	..
..	S:Q493R	S:Q493R	..	..	S:Q493R
..	S:G496S	..	..	..	..
..	S:Q498R	S:Q498R	S:Q498R	S:Q498R	S:Q498R
..	S:N501Y	S:N501Y	S:N501Y	S:N501Y	S:N501Y
..	S:Y505H	S:Y505H	S:Y505H	S:Y505H	S:Y505H
..	S:T547K	..	..	..	..

[a] Variant BA.1.617.2 was formerly known as Delta variant, mutations in the gene S (protein spike coding gene) were identified for Delta variant, and each Omicron sublineage (BA.1, BA.2, BA.4, BA.5 and BA.2.12.1).

purified using the Axygen AxyPrep MagPCR Clean-up beads (Axygen, MAG-PCR-CL-50), evaluated using the Agilent Fragment Analyzer automated electrophoresis system (Agilent, Santa Clara, CA.) and quantified through a Qubit HS dsDNA assay (Invitrogen, Waltham, MA.), according to the instructions given by the respective providers. For sequencing, pools were diluted following Illumina's standard protocol (Document # 1000000025416 v09), and paired-end 300 base pair length reads were generated using an Illumina MiSeq instrument (Illumina, San Diego, CA).

The bioinformatic pipeline that we implemented was originally developed by Gregory et al. [23] to solve the amalgamation of circulating lineages contained in wastewater samples which hinders the reconstruction of individual SARS-CoV-2 genomes and the identification of multiple nucleotide polymorphisms, insertion, and deletions events and downstream amino acid changes. This pipeline begins with a pre-processing step that includes low-quality reads and adapter trimming using Cutadapat, merging and dereplication of reads using VSEARCH tool, and finally, the mapping of reads against the reference genome of SARS-CoV-2 (NC_045512.2) using Bowtie2. SAM files obtained from the preprocessing step are used as the input for SAM Refiner, a CLI-based Python script that generates five outputs (Sample_unique_seqs.tsv, Sample_nt_calls.tsv, Sample_indels.tsv, Sample_covars.tsv and Sample_chim_rm.tsv) which as a summary not only report the variation calls but also their occurrence count and abundance [23]. Genotypic information of our samples was extracted from the "Sample_chim_rm.tsv" file as a constellation of linked mutations alongside their respective occurrence count and abundance [23]. Based on this data we assigned a lineage to the assessed samples.

Results

During the fourth wave of SARS-CoV-2 in Mexico, the presence of SARS-CoV-2 Delta and Omicron variants in wastewater samples was confirmed. As shown in Table 2, SARS-CoV-2 Delta variant was con-

Table 2
SARS-CoV-2 variant determination and distribution during the fourth SARS-CoV-2 pandemic wave in Mexico. Variant determination was performed by Thermo Fisher mutation panel protocol.

#	Sampling point	Collection date	MUTATION PANEL					Lineage
			L452R	T478K	E484Q	Q493R	P681R	
1	Querétaro	August 30, 2021	MUT	MUT	NA	NA	MUT	Delta
2	Mexico City	August 31, 2021	MUT	MUT	NA	NA	MUT	Delta
3	Aguascalientes	November 29, 2021	MUT	MUT	WT	WT	MUT	Delta
4	Aguascalientes	November 29, 2021	MUT	MUT	WT	WT	MUT	Delta
5	Ciudad Juárez	November 29, 2021	MUT	MUT	WT	WT	MUT	Delta
6	Monterrey	January 13, 2022	WT	MUT	NA	MUT	NA	Omicron
7	Celaya	January 13, 2022	KT	MUT	NA	MUT	NA	Omicron
8	Estado de México	January 13, 2022	WT	MUT	NA	MUT	NA	Omicron
9	San Luis potosi	January 13, 2022	WT	NA	MUT	NA	NA	N/D
10	Zacatecas	January 13, 2022	WT	MUT	NA	MUT	NA	Omicron
11	Tampico	January 13, 2022	WT	MUT	NA	MUT	NA	Omicron
12	Querétaro	January 13, 2022	WT	MUT	NA	MUT	NA	Omicron
13	Puebla	January 13, 2022	WT	MUT	NA	MUT	NA	Omicron
14	Monterrey	January 17, 2022	WT	MUT	NA	NA	NA	N/D
15	Aguascalientes	January 17, 2022	WT	MUT	NA	MUT	NA	Omicron
16	Puebla	January 17, 2022	WT	MUT	NA	MUT	NA	Omicron
17	Hidalgo	January 17, 2022	WT	MUT	NA	MUT	NA	Omicron
18	Guadalajara	January 17, 2022	WT	MUT	NA	MUT	NA	Orncron
19	Laguna	January 17, 2022	WT	MUT	NA	NA	NA	N/D
20	Sonora	January 17, 2022	WT	MUT	NA	MUT	NA	Omicron
21	Estado de México	January 17, 2022	WT	MUT	NA	MUT	NA	Omicron
22	Querétaro	January 17, 2022	WT	MUT	NA	MUT	NA	Omicron
23	Mexico City	January 17, 2022	WT	MUT	NA	MUT	NA	Omicron
24	Irapuato	January 17, 2022	WT	MUT	NA	MUT	NA	Omicron
25	Santa fe	January 17, 2022	WT	MUT	NA	MUT	NA	Omicron
26	San Luis Potosí	January 17, 2022	WT	MUT	NA	MUT	NA	Omicron
27	Mexico City	January 17, 2022	WT	NA	NA	MUT	NA	Orncron
28	Mexico City	January 17, 2022	WT	NA	NA	MUT	NA	Omicron
29	Mexico City	January 17, 2022	WT	NA	NA	MUT	NA	Omicron
30	Irapuato	January 17, 2022	WT	NA	NA	MUT	NA	Omicron
31	Monterrey	March 7, 2022	NA	NA	NA	NA	NA	N/D
32	San Luis Potosí	March 7, 2022	NA	NA	NA	NA	NA	N/D
33	Monterrey	March 14, 2022	WT	MUT	NA	MUT	NA	Omicron
34	Sinaloa	March 25, 2022	NA	NA	NA	NA	NA	N/D

Mut: the result indicates a mutated allele detection in the sample. WT: the result indicates a wild-type allele detection in the sample. NA: the result indicates "No Amplification" detection in the sample. N/D: indicate the sample was not determined by the mutation panel.
Six samples (between January and March 2022) were not confirmed by the panel mutation assay.

firmed in samples taken from Querétaro and Mexico City in late August, 2021, and in Aguascalientes and Ciudad Juárez campuses in November 2021, as significant surges in cases belonging to the fourth wave of infections in the country started to be reported. However, the Delta variant was displaced by the SARS-CoV-2 Omicron variant in January 2022. The Omicron variant became predominant from January to March 2022 until the end of the fourth wave. In six samples, for which lineages are denoted as N/D in Table 2, it was unable to identify the assessed mutations by the TaqMan SARS-CoV-2 Mutation Panel and circulating lineages could not be determined. To confirm previous variant discrimination, Omicron-positive samples were sequenced using the technique published by Smyth et al. [22] to identify the mutations located at the RBD region of the SARS-CoV-2 genome to determine the circulating lineage in these samples. As shown in Table 3, two sequenced samples (3-51 and 3-56) that were also assessed using the Thermo Fisher Mutation Panel showed a mutation profile that matched the characteristic constellation of mutations present in the Omicron sublineage BA.1 genome. Additionally, seven more samples taken from wastewater treatment plants around the Monterrey Metropolitan Area (2-21, 2-34, 3-41, 2-22, 3-27, 2-58, and 3-8), which were not assessed using the Thermo Fisher Mutation Panel also showed mutations that matched those in the Omicron sublineage BA.1 genome. As a curious result, in these samples a genomic variant that resembles the signature mutation F486V of sublineages BA.4 and BA.5 (that were officially reported 5 months after the collection of these samples) was found in up to 2% of the reads obtained by RBD amplicon sequencing. Meanwhile, reads containing the Omicron BA.1 mutations were up to 60% of the total, as shown in the Supplementary Information. Since not enough sequencing reads supported the presence of the F486V mutation, the genotype remained undetermined for that proportion of the analyzed amplicons and was denoted as WT in Table 3.

Discussion

WBE is a complementary method to more complex and lengthy survey approaches for monitoring the distribution and spreading of infectious diseases, pathogenic agents, and biological markers [24]. Over the SARS-CoV-2 pandemic evolution, WBE was used to understand the real-time distribution of the virus around the time and territories, target viral residues of SARS-CoV-2 which served as a biological marker of the pathogen, previous data correlated the presence of viral biomarker and concentration con with the rise of the confirmed cases of SARS-CoV-2 variants, as shown in previous work [25].

The most common problem in the COVID-19 pandemic was the limited epidemiological scope of the disease using traditional surveillance techniques. This was due to their application being limited to a small population and the lack of diagnostic capacity. To address this, we developed a method for the detection of SARS-CoV-2 in wastewater samples. This method can detect even a few copies of the virus in a fast and cost-effective manner. The method has been previously described and reported [25]. Additionally, considering the concern surrounding the new variants of SARS-CoV-2 and their clinical and epidemiological relevance, we developed a new method for the detection of VOC using RT-qPCR. This method utilizes specific primers and probes that recognize specific mutations found in the Omicron and Delta variants. The gold standard test for the identification of VOC is whole genome sequencing (WGS). Most of the studies in Mexico focused on sequencing clinical samples [26], to determine the prevalence of the SARS-CoV-2 VOC. In our study, we tried a different approach using RT-qPCR and the Amplicon sequencing of the RBD region, because of how expensive and inaccessible to the entire population is WGS. WGS has limitations in the number of specimens that can be processed and requires a high level of expertise for data analysis [27]. With the RBD region sequencing approach, it is less expensive and the bioinformatic analysis is more suitable to determine the SARS-CoV-2 variant in wastewater samples. The most common alternative for the detection of SARS-CoV-

Table 3
Summary of mutations found in the wastewater samples, where RBD amplicon sequencing was performed.

ID	Sample	Thermo Fisher	Sample origin	Receptor binding domain mutations											Lineage	Other mutations	Collection date
		Mutation panel		K417N	N440K	G446S	L452R/Q	S477N	T478K	E484A	F486V	Q493R	G496S	Q498R	N501Y	Y505H	T547K			
	3-13	NA	DE29: PTAR NORTE	WT	WT	WT	MUT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	DELTA	NA	Nov 21, 2021
	2-39	NA	DF47: PTAR DN	WT	WT	WT	MUT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	DELTA	NA	Dec 12, 2021
	3-17	NA	DF47: PTAR DN	WT	WT	WT	MUT	WT	MUT	WT	WT	WT	WT	WT	WT	WT	WT	DELTA	NA	Dec 12, 2021
	2-21	N/D	DF53: PTAR Norte	MUT	MUT	MUT	WT	MUT	MUT	MUT	WT	MUT	MUT	MUT	MUT	MUT	MUT	Omicron BA.1	F515I	Jan 03, 2022
	2-34	N/D	DF51:PTAR DN	MUT	MUT	MUT	WT	MUT	MUT	MUT	WT	MUT	MUT	MUT	MUT	MUT	MUT	Omicron BA.1	563fs	Jan 03, 2022
	3-41	NA	DF51: PTAR DN	MUT	MUT	MUT	WT	MUT	MUT	MUT	WT	MUT	MUT	MUT	MUT	MUT	MUT	Omicron BA.1	C538F, 557fs	Jan 03, 2022
	2-22	N/D	DF62: PTAR Cadereyta	MUT	MUT	MUT	WT	MUT	MUT	MUT	WT	MUT	MUT	MUT	MUT	MUT	MUT	Omicron BA.1	P561S	Jan 10, 2020
17	3-51	Omicron	Puebla	MUT	MUT	MUT	WT	MUT	MUT	MUT	WT	MUT	MUT	MUT	MUT	MUT	MUT	Omicron BA.1	F562F	Jan 13, 2022
	3-27	NA	DF57: PTAR DN	MUT	MUT	MUT	WT	MUT	MUT	MUT	WT	MUT	MUT	MUT	MUT	MUT	MUT	Omicron BA.1	K558N	Jan 17, 2022
19	3-56	Omicron	Aguascalientes	MUT	MUT	MUT	WT	MUT	MUT	MUT	WT	MUT	MUT	MUT	MUT	MUT	MUT	Omicron BA.1	530fs	Jan 17, 2022
	2-58	N/D	DF58: PTAR Norte	MUT	MUT	MUT	WT	MUT	MUT	MUT	WT	MUT	MUT	MUT	MUT	MUT	MUT	Omicron BA.1	L452L, A475P	Jan 17, 2022
	3-8	NA	DF87: PTAR Cadereyta	MUT	MUT	MUT	WT	MUT	MUT	MUT	WT	MUT	MUT	MUT	MUT	MUT	MUT	Omicron BA.1	NA	Jan 24, 2022

Mut: the result indicates a mutated allele detection in the sample. WT: the result indicates a wild-type allele detection in the sample. NA: the result indicates "No Amplification" detection in the sample. N/D: indicate the sample was not determined by the mutation panel.

2 variants is an analysis of the single-nucleotide polymorphisms in S protein-encoding genes via real-time RT-PCR. Among the advantages of this method are its cost-effectiveness, speed, and comparable accuracy to sequencing by next-generation sequencing [28]. However, the available kits, such as TaqPath COVID-19 RT-PCR, have been designed to be used in clinical samples. Identifies deletion in S gene (69/70 deletion) which allows for distinguishing between SARS-CoV-2 variants [29]. It is a multiplexed assay based on three specific sets to the ORF1ab, N gene, and S gene primer/probes of SARS-CoV-2 and is useful for identifying variants namely Alpha and Omicron BA.1. This method is based on S gene target failure.

The Omicron variant represented more than half (30/44) of samples analyzed in this study, and it was detected in January 2022 in the wastewater samples, displacing the Delta variant during the fourth wave in Mexico. The Delta variant was last detected in December 2021 with more prevalence in wastewater samples, contrary to clinical reports, where the Omicron variant detection in Mexico started in December 2021 [30]. However, the number of Omicron cases was not sufficient to be detected in wastewater samples but started to increase in January 2022 when the Omicron detection in wastewater started, which indicated a clear displacement of the Delta variant with the Omicron variant. While this study did not determine the limit of detection of the detection assay, commercial kits for SARS-CoV-2 surveillance in wastewater samples, including the one used here (SARS-CoV-2 RT-PCR Test, IDEXX, Westbrook, ME) have detection limits of around 10 copies per PCR reaction [31], which is consistent with previous surveillance efforts conducted by our team (unpublished data). This indicates that WBE methods can detect the circulation of SARS-CoV-2 variants shortly after their emergence in a given population, sometimes even before surges in clinical cases are reported, as demonstrated in our work in the Monterrey Metropolitan Area [25].

In this study, we used a commercially available RT-qPCR mutation detection panel, a commercial kit for clinical samples of Thermo Scientific, selecting five relevant mutations that can discriminate between the Omicron and Delta SARS-CoV-2 variants. Although this approach is not suitable when there is more than one variant type in the same sample, we were able to discriminate the variants in 28 wastewater samples, which is a limitation of the mutation detection panel. However, it is necessary to consider that the detection kit is used for the detection of variants in clinical samples. In three samples, identified as ND, in the lineage column of Table 3, there were only two allele discrimination detected, which is not sufficient to discriminate between Omicron and/or Delta variants. The lack of amplification could have been due to an MNP in the wastewater sample, and the RNA degradation might have influenced the lack of the amplification of the targets and also the scope of the detection to clinical samples, also the wastewater matrix has been proven to have several complications (genetic material degradation, sample dilution, population displacement in order to get a clearly and reliability information about outbreaks behaviors, however WBE also has been proved as a powerful tool to obtain very detailed information about the distribution of pathogens, even if the behaviors are similar to clinical data [32].

The present work confirmed 12 samples by RBD amplicon sequencing, to determine the presence of characteristic mutations for variant discrimination. Three of these sequenced samples corresponded to the Delta variant and were collected during November-December 2021. As previously reported during the fourth wave in Mexico, Delta variants were still detected in December 2021, meanwhile, the Omicron variants started to appear in wastewater samples in January 2022, coinciding with the clinical reports [33]. Additionally, this work was available to identify unexpected mutations in samples 3-51 and 3-56, both taken in January 2022, these mutations were found to belong to BA.4 and BA.5 lineages. However, it is important to notice that they were present at very low abundances in wastewater samples at that stage of the pandemic (Supplementary Table 1). The presence of these mutations does not affect the prevalence of the BA.1 variant as the predominant variant in the wastewater samples, although it does present at low abundance rate, nonsignificant indication of the presence of the other variants in the wastewater samples despite BA.1. Moreover, it is curious result the identification of these mutations present in the BA.4 and BA.5 subvariants in the wastewater samples, notice the mutations related to BA.4 y BA.5 were only officially announced on April 04, 2022 [34], when they were found in clinical samples, 3 months after the collection of both of these study samples. However, there are earlier reports that set the appearance of the sublineages BA.4 and BA.5 in South Africa in January and February 2022, respectively which align with our results [35]. While in sewage, one of the first studies to detect these Omicron sublineages in wastewater, Johnson et al. reported their appearance on April 18, 2022, in South Africa which it is inconsistent with our findings [36]. Until Omicron subvariant BQ.1, variant with the K444T, L452R, N460K, and F486V mutations [37], screening of mutation F486V was exclusive to BA.4 and BA.5 sublineages and it was used to confirm their presence in clinical and wastewater samples [38]. Changes in that locus of the SARS-CoV-2 had not been reported since the beginning of the pandemic until the onset of BA.4 and BA.5.

Other mutations not associated with a specific lineage were identified in eight of the sequenced samples (2-21, 2-34, 3-41, 2-22, 3-51, 3-27, 3-56, 2-58 samples) (Table 3). Three frameshift-causing mutations (530fs, 557fs, and 563fs) with no previous reports in the public databases were identified. A synonym substitution (F562F) was also detected in sample 3-51. This mutation was previously identified in collected air samples using targeted sequencing [39]. It was tagged as a rare variant not associated with any lineage. Also, synonym mutation L452L was identified in sample 2-58. There is a report where this mutation was detected in Omicron genomes from clinical samples during the end of 2021 and early 2022 which corresponds to our findings [40]. Furthermore, five rare nonsynonym substitutions: A475P, F515I, C538F, K558N, and P561S were found in samples 2-58, 2-21, 3-41, 3-27, and 2-22 respectively. The mutation C538F is not linked to any particular lineage and it has been identified with low frequency in genomes from lineages such as Delta, BA.1 and BA.2 lineage according to the GISAID database (Supplementary Information S2) [41]. Mutation K558N has been observed in genomes derived from Delta lineages but more recently in genomes from sublineages such as XBB. In addition, K558N has been identified in SARS-CoV-2 sequences derived from infected Malayan tigers who showed respiratory symptoms similar to humans [42]. Mutation F515I has not been linked to any specific lineage but, according to GISAID, it has been observed in a low frequency of genomes from lineages such as B.1.617.2, Omicron BA.1, BA.2, BA.4, and BA.5 including more recent sublineages as XBB and BN.1. Mutation A475P is also another rare variant that has been found in a very low number of genomes in the GISAID database and is not a defining mutation of any known lineage. A475P alongside other neighboring mutated residues integrates a cluster that has been associated with an improved anchorage of the RBD region to human receptor ACE2 [43]. Lastly, mutation P561S is a more common change found in SARS-CoV-2 genomes, but it is not linked to any VOC or VOI [44].

Conclusion

During the pandemic, the SARS-CoV-2 variants have gradually spread through the population, and observing a displacing phenomenon between variants, where the predominant variants are substituted by others adapted for faster distribution and avoiding epidemiological control mechanisms. Such a case was observed in the displacement of the Delta variant by the Omicron variant, between late 2021 and early 2022. At this time the presence of the Omicron variant was confirmed in clinical samples in Mexican territory and subsequently the predominant variant. This behavior was reflected in the data obtained from samples of wastewater using WBE surveillance.

Due to the mentioned above, WBE is a highly interesting tool for the surveillance of SARS-CoV-2 VOC. By using WBE, it was possible to de-

termine the presence and distribution dynamics of the variants along the fourth wave of the COVID-19 pandemic in Mexico territory, taking into account the limitations or variables that may affect the obtaining of results, inherent to the complexity of the wastewater as a working matrix. Additionally, it should be mentioned, the date and variant information were in concordance with the clinical tracking of COVID-19 variants. In correlation, the fluctuation of variant predominance can be observed over time. It was possible to observe in the last months of the year 2021, the predominant variant was Delta. Meanwhile, at the beginning of the year 2022 the dominant variant in the samples was Omicron BA.1. Furthermore, in a total of six samples, the identification of the virus variant was not achieved by commercial mutation panel (Table 2). Additionally, several rare mutations were identified in the RBD region amplicon, these mutations are no determinant to identify the lineage of the sample, such as A475P. However, it should be studied in more detail for the implication in modification of the virus infectivity and transmissibility. The obtained data indicate that the complexity of the wastewater as a source of information and the possible presence of more than one variant in each sample limits the spectrum of action for the identification tools used in this study. The present work demonstrates the long-term usefulness of WBE and potential long-term applications in future pandemics.

Declarations of competing interest

The authors have no competing interests to declare.

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Author contributions

Conceptualization, J.E.S.-H., M.A.O.-M., A.A-A.; methodology, M.A.O.-M., A.A.-A., K.O.R.-A., A.O.-C., K.D.R.-C., A.R.-Z., M.J.; writing—original draft preparation: M.A.O.-M., A.A.-A., K.O.R.-A., A.O.-C., writing—review and editing, J.E.S.-H., M.A.O.-M., A.A.-A., M.J., and R.P.-S.; supervision, J.E.S.-H., R.P.-S.; project administration, J.E.S.-H., M.A.O.-M., A.A.-A, and R.P.-S.; funding acquisition, J.E.S.-H., M.A.O.-M., and R.P.-S. All authors have read and agreed to the published version of the manuscript. M.A.O.-M., A.A-A. These authors contributed equally.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijregi.2023.11.005.

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