Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
26th Report
(Study period: October 2nd to October 6th, 2023)

Edited by:

- Giuseppina La Rosa, Giusy Bonanno Ferraro, Pamela Mancini, Carolina Veneri, Claudia Del Giudice, Marcello Iaconelli, Luca Lucentini (National Center for Water Safety, Istituto Superiore di Sanità)

- David Brandtner (Department of Infectious Diseases, Istituto Superiore di Sanità)

- Mauro Grigioni, Mirko Rossi (National Center for Innovative Technologies in Public Health, Istituto Superiore di Sanità)

- Elisabetta Suffredini (Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità

Main findings:

- During the week of 2 October to 6 October 2023, a total of 110 wastewater samples were collected from 16 Regions and 2 Autonomous Provinces (A.P.).
- Mutations characteristic of the Omicron variant were identified in 13 of these Regions/AP, while no sequencing data were collected from the remaining areas.
- Analysis of the sequences obtained by Sanger sequencing showed that 76% of the positive samples displayed amino acid substitutions typical of the Omicron XBB.1.5*/XBB.1.9* lineages, 12% had amino acidic substitutions of the Omicron XBB.2.3* lineage, 7% of the Omicron XBB.1.16*, 2% of the Omicron CH.1.1* lineage and the same percentage of the Omicron CM.7* lineage.

Introduction

On March 17th, 2021, the European Union Commission issued Recommendation 2021/472, encouraging Member States to establish a systematic surveillance of SARS-CoV-2 and its variants in wastewater by October 1st, 2021. In response to this recommendation, the Istituto Superiore di Sanità (ISS) started a series of "flash surveys". These surveys consist of monthly sampling campaigns carried out over short periods in different locations throughout Italy. The primary objective of these flash surveys is to gather supplementary information on SARS-CoV-2 variants in the population, complementing data obtained through clinical surveillance. The aim of this report is to summarise the results of the 26th national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from 2 to 6 October 2023.

Methodology

The 26th national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out in Italy from 2 to 6 October. The survey involved the collection of 110 sewage samples from 107 wastewater treatment plants (WTPs) located across 16 Regions and 2 Autonomous Provinces. Information on the WTPs participating in the SARS-CoV-2 surveillance in urban wastewater in Italy can be found on the ISS website[1]. The samples collected during the survey were processed and the viral concentration was determined by laboratories within the SARI network using the protocol "Sorveglianza di SARS-CoV-2 in reflui urbani - Protocollo progetto SARI - rev.3"[2]. Purified RNA extracts from the samples were delivered to ISS for variant detection.

For sequencing purposes, we employed a long-nested PCR assay covering approximately 1330 base pairs and spanning from amino acid residues 34 to 475 of the spike protein (PCR ID 1033/1034). After amplifying the target sequences, we used Sanger sequencing on individual samples.

For variant classification, we adopted a lineage classification based on 'outbreak.info'[3] rather than specifying sublineages. This choice was made because there are many sublineages that evolve rapidly, often converging on specific amino acid substitutions. In some cases, the differences between sublineages can be as small as a single nucleotide mutation in our target

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1 Surveillance of SARS-CoV-2 in urban wastewater in Italy 1° Report (Study period: 01 October 2021 - 31 March 2022) 8e5e2edb-bae0-f1b0-ee6e-08255c76484f (iss.it)

2 DOI 10.5281/zenodo.5758724.

3 https://outbreak.info/situation-reports, date: 05/12/2023

region, making a reliable assignation to sublineages, based solely on the mutations observed in the spike region, not feasible.

Results

Real Time qPCR

Out of the 103 samples analysed, a total of 94 (91.3%) tested positive for SARS-CoV-2 using the real-time RT-qPCR method employed for environmental surveillance (Table 1). The viral concentrations detected in these samples varied, ranging from 2.50E + 00 to 8.97E + 05 genome copies (g.c.) per liter of sewage.

Sanger Sequencing

Table 1 provides a summary of the results obtained from the long-nested PCR assay and sequencing methods. A total of 47 samples (43%) from 13 Regions/AP were successfully amplified using the long-nested PCR assay described above. Among these samples, high-quality sequences were obtained from 42 of them through Sanger sequencing, and five sequences were unsuccessful due to high signal noise resulting in partial sequence obtainment. Sanger sequencing confirmed that all the obtained sequences corresponded to the Omicron variant.

Analysis of wastewater samples revealed the presence of five SARS-CoV-2 lineages, as shown in Tables 1 and 2. Among these, the Omicron XBB.1.5*/XBB.1.9* lineages (which are not distinguishable in the sequenced region) were the most common, detected in 76% (32 samples) of the positive samples. In addition, Omicron XBB.2.3* was identified in 12% (5 samples), Omicron XBB.1.16* in 7% (3 samples), Omicron CH.1.1* in 2% (1 sample) and Omicron CM.7* in a further 2% (1 sample).

For ease of understanding, the mutations have been grouped into panels or "mutation packages". These are listed below:

- **Package A (assigned to the lineage Omicron XBB.1.5*/XBB.1.9*)** = V83A, G142D, DEL144, H146Q/H146K, Q183E, V213E, G252V, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K
- **Package B (assigned to the lineage Omicron XBB.1.5*/XBB.1.9*)**[4] = Q52H, V83A, G142D, DEL144, H146Q, Q183E, V213E, G252V, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, F456L, N460K
- **Package C (assigned to the lineage Omicron XBB.2.3*)** = V83A, G142D, DEL144, H146Q, Q183E, V213E, D253G, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K
- **Package D (assigned to the Omicron CH.1.1*)** = G142D, K147E, W152R, F157L, I210V, V213G, G257S, G339H, R346T, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, K444T, G446S, L452R, N460K

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[4] Package B may indicate the presence of the Omicron EG.5 sublineage. This last sublineage is characterised by the presence of the 'Q52H' mutation in approximately 93.5% and the 'F456L' mutation in about 96.2% of its sequences (https://outbreak.info/situation-reports, date: 12/12/2023).

- **Package E (assigned to the Omicron CM.7*) =** G142D, K147E, M153T, N164K, V213G, H245N, G257D, G339D, S371F, S373P, S375F, T376A, R403K, D405N, R408S, K417N, N440K, K444R, N450D, L452M, N460K
- **Package F (assigned to the Omicron XBB.1.16*) =** V83A, G142D, DEL144, H146Q, E180V, Q183E, V213E, G252V, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K

The Omicron XBB.1.5*/XBB.1.9* lineages were detected in 12 regions/A. P.: Abruzzo, Emilia-Romagna, Lazio, Liguria, Lombardia, Molise, Piemonte, Puglia, Sicilia, Veneto, and the autonomous provinces of Bolzano and Trento. Key mutations of the Omicron XBB.2.3* lineage were detected in three regions: Abruzzo, Friuli-Venezia Giulia and Liguria. Additionally, mutations of the Omicron XBB.1.16* lineage were observed in three regions: Liguria, Piemonte and Sicilia. The Omicron CH.1.1* lineage was found in only one sample from Molise, and Omicron CM.7* was identified in a single sample from Emilia-Romagna.

Table 1. PCR and sequencing results

	Sample ID	Region/A.P.	City	WTP	RT-qPCR (c.g./L)	Mutations found by Sanger sequencing (long PCR ID_1034)	SARS-CoV-2 lineages (Sanger sequencing)
1	22812		Pescara	Villa Carmine	< LOD		
2	22813		Pescara	Via Raiale	3,74E+02		
3	22814	Abruzzo	Chieti	S. Martino	4,13E+02		
4	22815		Teramo	Villa Pavone	1,51E+03	Package A + F456L	Omicron XBB.1.5*/XBB.1.9*
5	22816		L'Aquila	Pile	1,06E+03	Package C + N185D + L455F + F456L	Omicron XBB.2.3*
6	22787	Basilicata	Potenza	Tiera di Vaglio	2,89E+04		
7	22788		Matera	Pantano	4,67E+05		
103	23096	Campania	Napoli	Napoli Est	6,25E+03		
104	23097		Napoli	Napoli ovest	1,16E+04		
8	22729		Ferrara	Ferrara - Linea 1	2,29E+03		
9	22730		Ferrara	Ferrara - Linea 2	< LOD		
10	22731		Modena	Carpi	1,00E+03		
11	22809		Piacenza	Borgoforte	6,98E+03	Package B[a] + L455F	Omicron XBB.1.5*/XBB.1.9*
12	22810		Parma	Parma ovest	1,57E+04		
13	22811		Reggio Emilia	Mancasale	1,44E+04		
14	22829	Emilia-Romagna	Modena	Naviglio	1,14E+04		
15	22830		Ravenna - Forlì-Cesena	Ravenna	4,75E+04		
16	22831		Bologna	IDAR	5,95E+04	Package A + F456L	Omicron XBB.1.5*/XBB.1.9*
17	22832		Forlì-Cesena	Forlì	1,14E+04		
18	22833		Bologna	Imola	1,87E+04		
19	22834		Forlì-Cesena	Cesena	5,68E+04	Package A	Omicron XBB.1.5*/XBB.1.9*
20	22835		Rimini - Forlì-Cesena	S. Giustina	9,00E+03	Package E	Omicron CM.7*
21	22836	Friuli-Venezia Giulia	Pordenone	Cordenons	2,05E+04	Package C + N185D	Omicron XBB.2.3*
22	22837		Trieste	Servola	6,75E+03	Package C	Omicron XBB.2.3*
23	22684	Lazio	Viterbo	Viterbo - Strada Bagni	N.A.		
24	22685		Roma	Anzio - Colle Cocchino	N.A.		

#	ID	Region	Province	Location	Value	Mutations	Variant
25	22686		Latina	Aprilia (Via del Campo)	N.A.		
26	22687		Latina	Latina Loc Latina Est	N.A.		
27	22688		Roma	Guidonia - Ponte Lucano	N.A.	Package B[a] + L455F	Omicron XBB.1.5*/XBB.1.9*
28	22689		Roma	Velletri (LA CHIUSA-SORBO)	N.A.	Package B[a] + F157L + L452R[a]	Omicron XBB.1.5*/XBB.1.9*
29	22690		Roma	Pomezia - Via Cincinnato	N.A.	Package B[a]	Omicron XBB.1.5*/XBB.1.9*
30	22846		Roma	Civitavecchia Fiumaretta	2,50E+00		
31	22765		Savona	Savona	1,25E+04	Package B[a]	Omicron XBB.1.5*/XBB.1.9*
32	22766		Savona	Borghetto Santo Spirito	1,61E+05	Package A + F456L	Omicron XBB.1.5*/XBB.1.9*
33	22767		Genova	Pegli	6,94E+04		
34	22768		Genova	Voltri	6,71E+04	M.E. (partial sequence, from Q52H to G339H)[b]	Not assigned
35	22769		Genova	Quinto	4,14E+04	Package B[a]	Omicron XBB.1.5*/XBB.1.9*
36	22770		Genova	Rapallo	3,88E+04		
37	22771	Liguria	Genova	Sestri P	3,08E+04	Package B[a]	Omicron XBB.1.5*/XBB.1.9*
38	22772		Genova	Sturla	9,63E+04	Package A + L455F + F456L	Omicron XBB.1.5*/XBB.1.9*
39	22773		Genova	Darsena	2,92E+04	Package C	Omicron XBB.2.3*
40	22774		Genova	Punta Vagno Genova	4,46E+04	Package C	Omicron XBB.2.3*
41	22775		Genova	Valpolcevera	9,89E+03	Package F + S256L + F392L	Omicron XBB.1.16*
42	22776		La Spezia	La Spezia	1,27E+04	M.E. (partial sequence, from V83A to N460K)[b]	Not assigned
43	22808		Genova	Recco	4,08E+04		
44	22742		Milano	Milano Nosedo	1,46E+05		
45	22743		Milano	Milano San Rocco	2,36E+05	M.E. (partial sequence, from G142D to G446S)[b]	Not assigned
46	22744		Como	Como	1,04E+05	M.E. (partial sequence, from Q183E to G446S)[b]	Not assigned
47	22745		Pavia	Pavia	5,80E+04		
48	22746	Lombardia	Como - Lecco - Milano - Monza e della Brianza	Monza	1,13E+05	M.E. (partial sequence, from V83A to N460K)[b]	Not assigned
49	22747		Pavia	Vigevano	7,07E+04		
50	22760		Sondrio	Sondrio	2,35E+04		
51	22777		Bergamo	Bergamo	1,36E+04	Package A + L455F + F456L	Omicron XBB.1.5*/XBB.1.9*
52	22778		Cremona	Citta di Cremona	1,29E+04		

53	22780		Brescia	Verziano	2,51E+04	
54	22797		Pesaro-Urbino	Borgheria	2,37E+04	
55	22798	Marche	Pesaro-Urbino	Ponte Metauro	8,43E+03	
56	22799		Ancona	Zipa	2,75E+04	
57	22800		Ancona	Falconara	2,07E+04	
58	22817		Campobasso	Campobasso - San Pietro	9,16E+02	Package D + N185D + L455F + F456L	Omicron CH.1.1*
59	22818	Molise	Campobasso	Termoli - località Porto	3,33E+03	Package A + F456L	Omicron XBB.1.5*/XBB.1.9*
60	22819		Campobasso	Termoli - località Pantano Basso	< LOD		
61	22891		Bolzano	IDA Bolzano	4,98E+04	Package Bª	Omicron XBB.1.5*/XBB.1.9*
62	22892	A.P. Bolzano	Bolzano	IDA Merano	7,99E+04		
63	22893		Bolzano	IDA Termeno	7,36E+04	Package A	Omicron XBB.1.5*/XBB.1.9*
64	22736		Trento	Trento nord	3,99E+05	Package A + F456L	Omicron XBB.1.5*/XBB.1.9*
65	22737	A.P. Trento	Trento	Trento sud	2,65E+05		
66	22738		Trento	Rovereto	8,97E+05	Package A + F456L	Omicron XBB.1.5*/XBB.1.9*
68	22648		Torino	Castiglione Torinese	3,30E+04		
69	22649		Biella	Biella Nord	8,83E+03	Package A + F456L	Omicron XBB.1.5*/XBB.1.9*
70	22650		Biella	Biella Sud	8,13E+03		
71	22651	Piemonte	Novara	Novara	2,83E+04	Package F + F456L	Omicron XBB.1.16*
72	22720		Alessandria	Alessandria	4,78E+04	Package A	Omicron XBB.1.5*/XBB.1.9*
73	22721		Asti	Asti	4,20E+04		
74*	22722		Cuneo	Cuneo	5,75E+04	Package A + L455F + F456L	Omicron XBB.1.5*/XBB.1.9*
75	22655		Bari	Bari Est	< LOD		
76	22656		Bari	Bari Ovest	5,90E+02		
77	22657	Puglia	Taranto	Taranto Bellavista	7,32E+02	Package A + F456L	Omicron XBB.1.5*/XBB.1.9*
78	22658		Taranto	Taranto Gennarini	6,17E+02		
79	22659		Trapani	Trapani	3,26E+03		
80	22660		Trapani	Mazara del Vallo	1,96E+03		
81	22661	Sicilia	Trapani	Marsala	5,18E+03	Package A + F456L	Omicron XBB.1.5*/XBB.1.9*
82	22761		Ragusa	Modica	6,55E+03		
83	22762		Ragusa	Vittoria	2,30E+03		

#	ID	Region	Province	Location	Value	Package	Lineage
84	22763		Ragusa	Ragusa	4,15E+03		
85	22764		Caltanissetta	Gela Macchitella	1,75E+03		
86	22782		Catania	Pantano d'Arci	1,49E+04		
87	22783		Catania	Giarre	1,31E+04		
88	22784		Siracusa	Siracusa	1,30E+04		
89	22793		Palermo	Bagheria	1,49E+04	Package A + F456L	Omicron XBB.1.5*/XBB.1.9*
90	22794		Palermo	Acqua dei Corsari	1,10E+04		
91	22795		Palermo	Fondo Verde	1,17E+04		
92	22796		Caltanissetta	Caltanissetta e San Cataldo	3,42E+04	Package F + F456L	Omicron XBB.1.16*
105	23087		Firenze	Pisa Nord - S.Jacopo	<LOD		
106	23088		Firenze	Empoli Pagnana	<LOD		
107	23089	Toscana	Massa	Lavello 2	<LOD		
108	23090		Lucca	Viareggio	<LOD		
109	23091		Massa	Lavello 1	5,75E+03		
110	23092		Livorno	Rivellino	<LOD		
111	23209	Umbria	Perugia	Perugia Pian della Genna	1,07E+05		
93	22253		Padova	Padova Ca' Nordio - centro storico	5,32E+04		
94	22652		Padova	Padova Ca' Nordio - zip	1,09E+05	Package B[a] + F157L + L452R	Omicron XBB.1.5*/XBB.1.9*
95	22653		Padova	Padova Guizza	9,67E+04	Package A + L455F + F456L	Omicron XBB.1.5*/XBB.1.9*
96	22654		Padova	Abano Terme	7,46E+04	Package A + L455F + F456L	Omicron XBB.1.5*/XBB.1.9*
97	22663	Veneto	Treviso	Treviso	2,35E+03	Package A + L452R + L455F + F456L	Omicron XBB.1.5*/XBB.1.9*
98	22664		Venezia	Venezia Fusina	2,43E+03	Package B[a]	Omicron XBB.1.5*/XBB.1.9*
99	22665		Vicenza	Vicenza Casale	1,69E+03	Package B[a]	Omicron XBB.1.5*/XBB.1.9*
100	22733		Verona	Verona_collettore 1M	5,84E+04		
101	22734		Verona	Verona_collettore 3M	3,12E+04	Package B[a] + L452R	Omicron XBB.1.5*/XBB.1.9*
102	22735		Verona	Verona_collettore 8M	1,17E+04	Package B[a] + V90G + F157L + V320A	Omicron XBB.1.5*/XBB.1.9*

NA= not available

[a] Package B may indicate the presence of the Omicron EG.5 sublineage. This last sublineage is characterised by the presence of the 'Q52H' mutation in approximately 93.5% and the 'F456L' mutation in about 96.2% of its sequences (https://outbreak.info/situation-reports, date: 12/12/2023).

[b] Partial sequence due to mixed electropherograms and/or high signal noise; within brackets the region for which a sequence was provided.

Table 2. Sanger sequencing results

ID SAMPLES	Q52H	V83A	G142D	DEL 144	H146Q/H146K	K147E	W152R	M153T	F157L	N164K	E180V	Q183E	I210V	V213G	V213E	H245N	G252V	D253G	G257D	G257S	G339D	G339H	R346T	L368I	S371F	S373P	S375F	T376A	R403K	D405N	R408S	K417N	N440K	K444R	K444T	V445P	G446S	N450D	L452M	L452R	F456L	N460K	VARIANTS
4, 16, 19, 32, 38, 51, 59, 63, 64, 66, 69, 72, 74, 77, 81, 89, 95, 96, 97		X	X	X	X							X			X		X					X	X	X	X	X	X	X		X	X	X	X			X	X					X	Package A (Omicron XBB.1.5*/XBB.1.9*)
11, 27, 28, 29, 31, 35, 37, 61, 94, 98, 99, 101, 102	X	X										X			X		X					X	X	X	X	X	X	X		X	X	X	X			X	X				X		Package B (Omicron XBB.1.5*/XBB.1.9*)
5, 21, 22, 39, 40		X	X	X	X							X			X		X	X				X	X	X	X	X	X	X		X	X	X	X			X	X						Package C (Omicron XBB.2.3*)
58		X				X	X		X		X		X							X		X	X	X	X	X	X			X		X	X		X		X			X		X	Package D (Omicron CH.1.1*)
20		X					X	X	X	X			X		X	X		X			X				X	X	X	X		X	X	X	X			X		X	X				Package E (Omicron CM.7*)
41, 71, 92		X	X	X						X	X				X		X					X	X	X	X	X		X		X	X	X	X			X	X					X	Package F (Omicron XBB.1.6*)

Limitations of the study

The geographical and population coverage of this flash survey is not representative of the entire territory of the country as it only covers 18 out of 21 of the Italian Regions/Autonomous Provinces. It is important to highlight that the employment of molecular analytical methods in complex environmental matrices such as wastewater can be challenging due to a number of factors. These include low virus concentration, insufficient analytical recovery and/or PCR inhibitors. Consequently, both the detection/quantification and the PCR amplification required for the sequencing may produce false negatives, making it difficult to achieve molecular characterization and variant detection for all samples. In addiction, obtainment of partial sequences from the spike region does not provide conclusive results for sublineage assignment. Our decision to adopt a broader lineage classification from 'outbreak.info' for variant classification, rather than specifying sublineage assignments, was influenced by the rapid evolution of numerous sublineages, often with minor differences, that hampered the reliable assignation to sublineages based solely on mutations observed in the spike region.

Conclusions and final considerations

This report is part of a monthly series focusing on SARS-CoV-2 and its variants in wastewater samples in Italy, in accordance with the EU Commission Recommendation 2021/472. The primary objective is to provide additional information on SARS-CoV-2 variants in the population, complementing data obtained through clinical surveillance. The results of this survey indicate that the Omicron variant is the sole SARS-CoV-2 variant in Italy, with the Omicron XBB.1.5*/XBB.1.9* lineages being the most prevalent. The Omicron XBB.2.3*, Omicron XBB.1.16*, Omicron CH.1.1* and Omicron CM.7* lineages have also been detected. The sequencing of SARS-CoV-2 in wastewater samples provides valuable additional information alongside the sequencing of clinical cases. This approach provides a more complete and accurate understanding of the circulating variants in the country, contributing to a better characterization of the spread and evolution of this virus.

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- Umbria: Giovanni Santoro (Regione Umbria), Ermanno Federici, Maya Petricciuolo, Sofia Barigelli (Laboratorio Microbiologia Applicata e Ambientale, DCBB Università di Perugia);
- Veneto: Francesca Russo, Gisella Pitter, Vanessa Groppi (Regione Veneto); Franco Rigoli, Marco Zampini (ARPAV - Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto); Tatjana Baldovin, Irene Amoruso (Università di Padova);
- P.A. Bolzano: Lorella Zago (P.A. Bolzano); Alberta Stenico, Anna-Maria Prast (A.P.P.A. Agenzia provinciale per l'ambiente e la tutela del clima, Laboratorio biologico)
- P.A. Trento: Francesco Pizzo; Andrea Anselmo, Elena Mengon (P. A. Trento) (P.A. Trento); Maria Cadonna, Mattia Postinghel (ADEP SGI PAT), Francesca Cutrupi, Paola Foladori, Serena Manara (UNITN – Università di Trento).