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**Flash survey on SARS-CoV-2 variants in urban wastewater in Italy**  
**27th Report**  
**(Study period: November 6<sup>th</sup> to November 10<sup>th</sup>, 2023)**

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**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 6 November to 10 November 2023, a total of 97 wastewater samples were collected from 15 Regions and 2 Autonomous Provinces (A.P.).
- Mutations characteristic of the Omicron variant were identified in 9 of these Regions/AP, while no sequencing data were collected from the remaining areas.
- The analysis of the sequences obtained by Sanger sequencing showed that 96% of the positive samples displayed amino acid substitutions that are typical of the Omicron XBB.1.5\*/XBB.1.9\* lineages, while the remaining 4% had mutations that are characteristic 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 27th national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from 6 to 10 November 2023.

## Methodology

The 27th national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out in Italy from 6 to 10 November 2023. The survey involved the collection of 97 sewage samples from 92 wastewater treatment plants (WTPs) located across 15 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<sup>1</sup>. 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"<sup>2</sup>. 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'<sup>3</sup> 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 region, making a reliable assignment to sublineages, based solely on the mutations observed in the spike region, not feasible.

<sup>1</sup> 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)

<sup>2</sup> DOI 10.5281/zenodo.5758724.

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

## Results

### *Real Time qPCR*

Out of the 95 samples analysed, a total of 88 (92.6%) 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 2.45E + 02 to 6.78E + 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 29 samples (31%) from 9 Regions/AP were successfully amplified using the long-nested PCR assay described above. Among these samples, high-quality sequences were obtained from 26 of them through Sanger sequencing, and 3 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 two 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 96% (25 samples) of the positive samples. In addition, Omicron CM.7\* was identified in 4% (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\*)<sup>4</sup>** = 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 Omicron CM.7\*)** = G142D, K147E, M153T, N164K, V213G, H245N, G257D, G339D, S371F, S373P, S375F, T376A, R403K, D405N, R408S, K417N, N440K, K444R, N450D, L452M, N460K

The Omicron XBB.1.5\*/XBB.1.9\* lineages were detected in nine regions/A.P.: Abruzzo, Emilia-Romagna, Friuli Venezia Giulia, Liguria, Lombardia, Puglia and Veneto, and the autonomous provinces of Bolzano and Trento. Key mutations of the Omicron CM.7\* lineage were found in one region (Lombardia).

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<sup>4</sup> 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 93.2% and the 'F456L' mutation in about 96.2% of its sequences (<https://outbreak.info/situation-reports>, date: 22/12/2023).

**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	Abruzzo	Chieti	S. Martino	3,79E+02		
2		Pescara	Via Raiale	1,03E+03	Package B <sup>a</sup> + F157L + L452R	Omicron XBB.1.5/1.9*
3		Pescara	Villa Carmine	7,61E+02		
4		Teramo	Villa Pavone	1,22E+03	Package A + L455F + F456L	Omicron XBB.1.5/1.9*
5		L'Aquila	Pile	9,42E+02	Package B <sup>a</sup> + L455F	Omicron XBB.1.5/1.9*
6	Basilicata	Potenza	Tiera di Vaglio	3,78E+04		
7		Matera	Pantano	1,78E+04		
8	Emilia-Romagna	Ferrara	Ferrara - Linea 1	1,94E+04		
9		Ferrara	Ferrara - Linea 2	<LOD		
10		Modena	Carpi	1,87E+04	M.E. <sup>b</sup>	Not assigned
11		Ravenna - Forlì-Cesena	Ravenna	1,23E+05		
12		Bologna	IDAR	1,96E+05	Package B <sup>a</sup>	Omicron XBB.1.5/1.9*
13		Ravenna	Faenza	5,13E+05	Package A + P217S + F456L	Omicron XBB.1.5/1.9*
14		Bologna	Imola	1,30E+05	Package B <sup>a</sup> + L455F	Omicron XBB.1.5/1.9*
15		Modena	Naviglio	1,58E+04	Package A + F456L	Omicron XBB.1.5/1.9*
16		Forlì-Cesena	Cesena	6,78E+05	Package A	Omicron XBB.1.5/1.9*
17		Rimini - Forlì-Cesena	S. Giustina	1,77E+05	Package B <sup>a</sup> + L452R	Omicron XBB.1.5/1.9*
98		Parma	Parma ovest	1,60E+03		
100		Piacenza	Borgoforte	6,80E+03		
101		Reggio Emilia	Mancasale	3,88E+03		
18	Friuli-Venezia Giulia	Pordenone	Cordenons	4,18E+04	Package A + L452R + L455F + F456L	Omicron XBB.1.5/1.9*
19		Udine	Udine	1,79E+05		
20		Trieste	Servola	1,77E+05	Package B <sup>a</sup> + F157L + L452R	Omicron XBB.1.5/1.9*
21	Lazio	Roma	Civitavecchia Fiumaretta	<LOD		
28	Liguria	La Spezia	La Spezia	8,18E+03	Package A + L452R + L455F + F456L	Omicron XBB.1.5/1.9*

29	23286		Savona	Savona	2,39E+04		
30	23287		Savona	Borghetto Santo Spirito	1,26E+04		
31	23288		Imperia	Imperia	1,39E+04		
32	23289		Imperia	Sanremo - località Capo Verde	3,49E+04		
33	23290		Genova	Pegli	7,59E+04		
34	23291		Genova	Voltri	1,06E+04	Package B <sup>a</sup> + G184S + L455F	Omicron XBB.1.5/1.9*
35	23292		Genova	Quinto	2,00E+04	Package B <sup>a</sup> + L455F	Omicron XBB.1.5/1.9*
36	23293		Genova	Rapallo	1,86E+04		
37	23294		Genova	Sestri P	8,60E+03		
38	23295		Genova	Sturla	7,69E+03		
39	23296		Genova	Darsena	6,33E+03		
40	23297		Genova	Punta Vagno Genova	1,41E+04		
41	23298		Genova	Valpolcevera	2,99E+03		
99	23398		Genova	Recco	4,38E+03		
42	23308		Milano	Milano Nosedo	8,60E+03		
43	23309		Milano	Milano San Rocco	7,69E+03		
44	23310		Como	Como	6,33E+03	Package B <sup>a</sup> + L455F	Omicron XBB.1.5/1.9*
45	23311		Pavia	Pavia	1,41E+04		
46	23312	Lombardia	Como - Lecco - Milano - Monza e della Brianza	Monza	2,99E+03		
47	23313		Pavia	Vigevano	4,38E+03		
49	23331		Bergamo	Bergamo	5,05E+03	Package C	Omicron CM.7*
50	23333		Cremona	Citta di Cremona	1,27E+04		
51	23337		Brescia	Verziano	3,45E+03		
52	23374		Pesaro-Urbino	Borgheria	2,89E+04		
53	23375	Marche	Pesaro-Urbino	Ponte Metauro	1,58E+04		
54	23377		Ancona	Zipa	4,34E+04		
55	23379		Ancona	Falconara	3,33E+04		
56	23367	Molise	Campobasso	Campobasso - San Pietro	<LOD		
57	23368		Campobasso	Termoli - località Porto	<LOD		

58	23369		Campobasso	Termoli - località Pantano Basso	<LOD		
59	23450	A.P. Bolzano	Bolzano	IDA Bolzano	7,28E+04	Package A + L455F + F456L	Omicron XBB.1.5/1.9*
60	23451		Bolzano	IDA Merano	8,69E+04	Package B <sup>a</sup> + F157L + L452R	Omicron XBB.1.5/1.9*
61	23452		Bolzano	IDA Termeno	1,07E+05	M.E. (partial sequence, from G252V to N460K) <sup>b</sup>	Not assigned
62	23232	A.P. Trento	Trento	Trento nord	1,44E+05		
63	23233		Trento	Trento sud	5,33E+05	Package A + F157L + L452R + L455F + F456L	Omicron XBB.1.5/1.9*
64	23234		Trento	Rovereto	6,38E+05	Package A + L455F + F456L	Omicron XBB.1.5/1.9*
65	23214	Piemonte	Torino	Castiglione Torinese	1,99E+04		
66	23215		Biella	Biella Nord	3,30E+04		
67	23216		Biella	Biella Sud	4,03E+04		
68	23217		Novara	Novara	4,15E+04		
69	23265		Alessandria	Alessandria	1,60E+04		
70	23266		Asti	Asti	1,88E+04		
71	23267		Cuneo	Cuneo	4,53E+04		
72	23225	Puglia	Bari	Bari Est	<LOD		
73	23226		Bari	Bari Ovest	4,73E+02		
74	23227		Taranto	Taranto Bellavista	<LOD		
75	23228		Taranto	Taranto Gennarini	2,45E+02	Package A + V70A + F157L + L455F + F456L	Omicron XBB.1.5/1.9*
76	23236	Sicilia	Trapani	Mazara del Vallo	8,67E+02		
77	23281		Ragusa	Modica	1,93E+03		
78	23282		Ragusa	Vittoria	3,03E+03		
79	23283		Ragusa	Ragusa	5,60E+03		
80	23284		Caltanissetta	Gela Macchitella	6,75E+02		
81	23339		Palermo	Acqua dei Corsari	1,09E+04		
82	23340		Palermo	Fondo Verde	4,56E+03		
83	23341		Caltanissetta	Caltanissetta e San Cataldo	1,75E+04		
96	23381		Catania	Pantano d'Archi	1,09E+04		
97	23382		Catania	Giarre	1,41E+04		
102	23235		Trapani	Trapani-Paceco-Erice-Casa Santa	N.A.		

103	23237		Marsala	Marsala	N.A.		
104	24239	Toscana	Pisa	Pisa	5,23E+03		
105	24240		Lucca	Lucca	5,23E+03		
84	23273	Umbria	Perugia	Perugia - Pian della Genna	6,94E+04		
85	23221	Veneto	Padova	Padova Ca' Nordio - centro storico	4,06E+05		
86	23222		Padova	Padova Ca' Nordio - zip	4,41E+05	Package A + F456L	Omicron XBB.1.5/1.9*
87	23223		Padova	Padova Guizza	2,36E+05	Package B <sup>a</sup> + L455F	Omicron XBB.1.5/1.9*
88	23224		Padova	Abano Terme	3,95E+05	M.E. (partial sequence, from G142D to N4560K) <sup>b</sup>	Not assigned
89	23238		Treviso	Treviso	3,80E+03	Package B <sup>a</sup> + L455F	Omicron XBB.1.5/1.9*
90	23239		Venezia	Venezia Fusina	3,34E+03	Package A + F456L	Omicron XBB.1.5/1.9*
91	23240		Vicenza	Vicenza Casale	1,45E+03		
92	23323		Verona	Verona_collettore 1M	2,89E+03		
93	23324		Verona	Verona_collettore 3M	6,43E+03	Package B <sup>a</sup> + L452R	Omicron XBB.1.5/1.9*
94	23325		Verona	Verona_collettore 8M	1,16E+04		

NA= not available

<sup>a</sup> 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 93.2% and the 'F456L' mutation in about 96.2% of its sequences (<https://outbreak.info/situation-reports>, date: 22/12/2023).

<sup>b</sup> 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	M153T	N164K	Q183E	V213G	V213E	H245N	G252V	G257D	G339D	G339H	R346T	L368I	S371F	S373P	S375F	T376A	R403K	D405N	R408S	K417N	N440K	K444R	V445P	G446S	N450D	L452M	F456L	N460K	VARIANTS		
4, 13, 15, 16, 18, 28, 59, 63, 64, 75, 86, 90																																					Package A (Omicron XBB.1.5*/XBB.1.9*)
2, 5, 12, 14, 17, 20, 34, 35, 44, 60, 87, 89, 93																																					Package B (Omicron XBB.1.5*/XBB.1.9*)
49																																				Package C (Omicron CM.7*)	



## 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 16 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 addition, 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 assignment 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. It has also detected the Omicron CM.7\* lineage. 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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