Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
23rd Report
(Study period: July 3ʳᵈ to July 7ᵗʰ, 2023)

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Main findings:

- During the week of 3 July to 7 July, 2023, a total of 122 wastewater samples were collected from 15 Regions and 2 Autonomous Provinces (A.P.).
- Mutations characteristic of the Omicron variant were identified in 10 of these Regions/AP, while no sequencing data was obtained from the remaining areas.
- Analysis of the sequences obtained by Sanger sequencing showed that 41% of the positive samples had amino acid substitutions belonging to the Omicron CM.7* lineage. Additionally 32% displayed mutations linked to the Omicron XBB.1.5*/1.9* lineage, while 18% were attributed to the Omicron XBB.1.16* lineage. A further 9% were associated with the Omicron XBB.2.3* 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. Responding to this recommendation, the Istituto Superiore di Sanità (ISS) initiated a series of "flash surveys". These surveys involve monthly sampling campaigns conducted at various locations across Italy over a short period of time. 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 summarize the findings from the 23rd national Flash Survey on SARS-CoV-2 variants in wastewater samples in Italy, which was conducted from July 3rd to July 7th, 2023.

Methodology

During the period from 3 July to 7 July, 2023, the 23rd national Flash Survey on SARS-CoV-2 variants in wastewater samples was conducted in Italy. The survey involved the collection of 122 sewage samples from 117 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[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]. The purified RNAs extracted from the samples were then sent to ISS for variant detection.

For sequencing purposes, we used 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 amplification of the target sequences, Sanger sequencing was performed 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 region, making

reliable sublineage assignation, on the basis solely of mutations observed in the spike region, not feasible.

Results

Real Time qPCR

Out of the 122 samples analysed, a total of 89 (73.0%) tested positive for SARS-CoV-2 using the real-time RT-qPCR method used for environmental surveillance (Table 1). The viral concentrations detected in these samples varied, ranging from 8.46E+01 to 2.95E+05 genome copies (g.c.) per liter of wastewater.

Sanger Sequencing

Table 1 provides a summary of the results obtained from the real-time PCR, long-nested PCR and sequencing. A total of 23 samples (18.9%) from 10 Regions/AP were successfully amplified using the long-nested PCR assay described above. Of these samples, 22 yielded high-quality sequences by Sanger sequencing, and one sequence was unsuccessful due to noisy sequencing signal. All the obtained Sanger sequences were assigned to the Omicron variant.

Analysis of the wastewater samples revealed the presence of four SARS-CoV-2 lineages, as shown in Tables 1 and 2. Among these, the Omicron CM.7* lineage was prevalent, detected in 41% (9 samples) of the positive samples. In addition, the Omicron XBB.1.5*/XBB 1.9* lineages (which are not distinguishable in the sequenced region) were detected in the 32% of the positive samples (7 samples), Omicron XBB.1.16* in 18% (4 samples) and Omicron XBB.2.3* in 9% (2 samples).

For ease of understanding, the mutations have been grouped into panels or "mutation packages" as follows:

- **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.1.16*)** = V83A, G142D, DEL144, H146Q, E180V, Q183E, V213E, G252V, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, 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 lineage 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 4 regions/A. P. (Liguria, Lombardia, Puglia and Veneto). Key mutations of lineage Omicron XBB.1.16* were observed in 3 regions/A.P. (Lazio, Liguria and Marche). Key mutations of the Omicron XBB.2.3* lineage were found in 2 regions (Liguria and P.A. Trento). Finally, the Omicron CM.7* lineage was detected in 7 regions (Abruzzo, Lazio, Liguria, Lombardia, Marche, Puglia and Sicilia).

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
115	21476		Pescara	Villa Carmine	< LOD	Package D	Omicron CM.7*
116	21475		Pescara	Via Raiale	< LOD		
117	21474	Abruzzo	Chieti	S. Martino	< LOD		
118	21478		L'Aquila	Pile	9,61E+02		
119	21477		Teramo	Villa Pavone	< LOD		
1	21461	Basilicata	Potenza	Tiera di Vaglio	3,61E+04		
2	21462		Matera	Pantano	2,81E+04		
3	21452		Napoli	Napoli EST	1,18E+03		
4	21453	Campania	Napoli	Napoli OVEST - Ingresso Principale	4,13E+03		
5	21454		Napoli	Napoli OVEST - ex ingresso Camaldoli	2,89E+03		
6	21370		Ferrara	Ferrara - Linea 1	3,50E+03		
7	21373		Ferrara	Ferrara - Linea 2	< LOD		
8	21377		Modena	Carpi	< LOD		
9	21424		Ravenna - Forlì-Cesena	Ravenna	1,01E+05		
10	21425		Bologna	Imola	4,25E+03		
11	21428		Modena	Naviglio	1,53E+04		
12	21429	Emilia-Romagna	Bologna	IDAR	1,78E+04		
13	21431		Forlì-Cesena	Forlì	9,00E+03		
14	21432		Forlì-Cesena	Cesena	5,00E+03		
15	21433		Rimini - Forlì-Cesena	S. Giustina	5,00E+03		
106	21451		Reggio Emilia	Mancasale	2,19E+03		
107	21449		Piacenza	Borgoforte	< LOD		
108	21450		Parma	Parma ovest	2,15E+03		
113	21632		Pordenone	Cordenons	1,25E+05		

114	21634	Friuli Venezia Giulia	Trieste	Servola	1,79E+05		
16	21320		Roma	Civitavecchia Fiumaretta	< LOD		
17	21332		Viterbo	Viterbo - Strada Bagni	6,83E+03	PACKAGE D	Omicron CM.7*
18	21333		Latina	Aprilia (Via del Campo)	4,30E+03	PACKAGE B	Omicron XBB.1.16*
19	21334		Roma	Anzio - Colle Cocchino	3,88E+03		
20	21335	Lazio	Latina	Latina Loc Latina Est	6,53E+03		
21	21336		Roma	Pomezia - Via Cincinnato	1,64E+03	PACKAGE B	Omicron XBB.1.16*
22	21337		Roma	Velletri (LA CHIUSA-SORBO)	< LOD		
23	21338		Roma	Guidonia - Ponte Lucano	2,08E+03		
123	21498		Roma	Roma Nord	< LOD		
124	21497		Roma	Roma Est (linea 1 + linea 2)	7,60E+02		
24	21387		Savona	Savona	4,99E+04		
25	21388		Genova	Pegli	2,02E+04	PACKAGE B	Omicron XBB.1.16*
26	21389		Genova	Voltri	1,48E+05		
27	21390		Genova	Quinto	2,95E+05	PACKAGE A	Omicron XBB.1.5*/XBB.1.9*
28	21391		Genova	Rapallo	6,09E+04		
29	21392		Genova	Sestri P	2,46E+04		
30	21393		Genova	Sturla	4,55E+04		
31	21394		Genova	Darsena	6,00E+04		
32	21395	Liguria	Genova	Punta Vagno Genova	7,95E+04	PACKAGE A (partial sequence, from V213E to N460K)[a]	Omicron XBB.1.5*/XBB.1.9*
33	21396		Genova	Valpolcevera	3,37E+04		
34	21397		Genova	Valpolcevera	4,13E+04		
35	21398		Imperia	Imperia	4,34E+04		
36	21399		Imperia	Sanremo - località Capo Verde	4,38E+04		
37	21400		La Spezia	La Spezia	9,14E+04	PACKAGE C + K444R	Omicron XBB.2.3*
38	21401		Savona	Borghetto Santo Spirito	8,46E+01	PACKAGE D	Omicron CM.7*
109	21934		Genova	Recco	< LOD		
39	21402	Lombardia	Bergamo	Bergamo	8,75E+02	PACKAGE A	Omicron XBB.1.5*/XBB.1.9*
40	21403		Cremona	Citta di Cremona	< LOD		

41	21406		Brescia	Verziano	1,03E+03	
98	21835		Como	Como	< LOD	
99	21833		Milano	Milano Nosedo	< LOD	
100	21834		Milano	Milano San Rocco	< LOD	
101	21836		Pavia	Pavia	< LOD	
102	21837		Como - Lecco - Milano - Monza e della Brianza	Monza	< LOD	PACKAGE D	Omicron CM.7*
125	21620		Sondrio	Sondrio	< LOD	
42	21410		Pesaro-Urbino	Borgheria	4,04E+03	PACKAGE D	Omicron CM.7*
43	21411	Marche	Pesaro-Urbino	Ponte Metauro	1,77E+03	
44	21412		Ancona	Zipa	1,87E+04	
45	21413		Ancona	Falconara	1,32E+03	PACKAGE B	Omicron XBB.1.16*
120	21479		Campobasso	Campobasso - San Pietro	< LOD	
121	21480	Molise	Campobasso	Termoli - località Porto	< LOD	
122	21481		Campobasso	Termoli - località Pantano Basso	< LOD	
46	21455		Bolzano	IDA Bolzano	4,31E+03	
47	21456	P.A. Bolzano	Bolzano	IDA Merano	3,68E+03	
48	21457		Bolzano	IDA Termeno	1,60E+03	M.E.[b]	
49	21328		Trento	Trento nord	9,75E+03	PACKAGE C	Omicron XBB.2.3*
50	21329	P.A. Trento	Trento	Trento sud	3,22E+04	
51	21330		Trento	Rovereto	1,91E+04	
58	21350		Cuneo	Cuneo	6,18E+02	
52	21301		Torino	Castiglione Torinese	7,50E+02	
53	21302		Biella	Biella Nord	2,00E+03	
54	21303	Piemonte	Biella	Biella Sud	< LOD	
55	21304		Novara	Novara	< LOD	
56	21348		Alessandria	Alessandria	8,90E+02	
57	21349		Asti	Asti	< LOD	
73	21385	Puglia	Foggia	Manfredonia	2,64E+03	PACKAGE D	Omicron CM.7*
74	21386		Foggia	Cerignola	9,14E+02	

59	21305		Bari	Altamura	3,34E+02		
60	21318		Taranto	Taranto Bellavista	< LOD	PACKAGE D	Omicron CM.7*
61	21319		Taranto	Taranto Gennarini	2,51E+02		
62	21323		Bari	Bari Est	< LOD		
63	21324		Bari	Bari Ovest	< LOD		
64	21325		Bari	Molfetta	9,86E+02	PACKAGE A	Omicron XBB.1.5*/XBB.1.9*
65	21331		Bari	Bitonto	< LOD	PACKAGE D	Omicron CM.7*
66	21342		Barletta-Andria-Trani	Bisceglie	4,45E+03		
67	21343		Barletta-Andria-Trani	Barletta	1,85E+03		
68	21344		Barletta-Andria-Trani	Andria	8,02E+03		
69	21345		Barletta-Andria-Trani	Trani	2,66E+03		
70	21346		Lecce	Lecce	< LOD		
71	21383		Brindisi	Brindisi Fiume Grande	3,86E+03		
72	21384		Foggia	Foggia	3,06E+03		
75	21354		Trapani	Trapani	1,40E+03		
76	21355		Trapani	Mazara del Vallo	1,14E+03		
77	21356		Trapani	Marsala	1,18E+03		
78	21371		Ragusa	Modica	2,00E+03		
79	21372		Ragusa	Vittoria	1,31E+04		
80	21374		Ragusa	Ragusa	8,75E+02		
81	21375		Caltanissetta	Gela Macchitella	5,35E+03		
82	21376	Sicilia	Messina	Mili Marina	1,50E+03		
83	21378		Messina	Mili Marina	2,65E+03		
110	21407		Catania	Pantano d'Arci	1,44E+04		
111	21408		Catania	Giarre	1,48E+04		
112	21409		Siracusa	Siracusa	1,49E+04		
84	21414		Agrigento	Agrigento	9,47E+03		
85	21415		Enna	Enna	4,30E+03		
86	21416		Palermo	Acqua dei Corsari	8,82E+03		
87	21417		Palermo	Fondo Verde	6,68E+03		

88	21418		Caltanissetta	Caltanissetta e San Cataldo	7,08E+03	PACKAGE D	Omicron CM.7*
89	21369	Umbria	Perugia	Perugia - Pian della Genna	6,49E+03		
90	21321	Venet	Padova	Padova Ca' Nordio - centro storico	8,83E+03	PACKAGE A	Omicron XBB.1.5*/XBB.1.9*
91	21322		Padova	Padova Ca' Nordio - zip	3,78E+03		
92	21339		Treviso	Treviso	< LOD		
93	21340		Venezia	Venezia Fusina	1,18E+04		
94	21341		Vicenza	Vicenza Casale	< LOD		
95	21380		Verona	Verona_collettore 1M	7,39E+03	PACKAGE A (partial sequence, from H146Q to N460K)[a]	Omicron XBB.1.5*/XBB.1.9*
96	21381		Verona	Verona_collettore 3M	< LOD	PACAGE A + E180V	Omicron XBB.1.5*/XBB.1.9*
97	21382		Verona	Verona_collettore 8M	< LOD		

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

[b]M.E. mixed electropherograms (noisy sequencing signal)

Table 2. Sanger sequencing results

ID SAMPLES	V83A	G142D	DEL 144	H146Q/H146K	K147E	M153T	N164K	E180V	Q183E	V213G	V213E	H245N	G252V	D253G	G257D	G339D	G339H	R346T	L368I	S371F	S373P	S375F	T376A	R403K	D405N	R408S	K417N	N440K	K444R	V445P	G446S	N450D	L452M	N460K	VARIANTS
27, 32, 39, 64, 90, 95, 96	■	■	■	■					■		■		■				■	■	■	■	■	■	■		■	■	■	■		■	■			■	**Package A (Omicron XBB.1.5*/XBB.1.9*)**
18, 21, 25, 45	■	■	■	■				■	■	■	■		■				■	■	■	■	■	■	■		■	■	■	■		■	■			■	**Package B (Omicron XBB.1.16)**
37, 49	■	■	■	■					■		■		■	■			■	■	■	■	■	■	■		■	■	■	■		■	■			■	**Package C (Omicron XBB.2.3*)**
17, 38, 42, 60, 65, 73, 88, 102, 115		■			■	■	■			■		■			■	■		■		■	■	■	■		■	■	■	■	■	■		■	■		**Package D (Omicron CM.7*)**

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Limitations of the study

The geographic and population coverage of this flash survey was not comprehensive, as it included 17 out of 21 of the Italian Regions/Autonomous Provinces.

It is important to note that the molecular analytical methods used for complex environmental matrices, such as wastewater, can be challenged by factors such as low viral concentrations, insufficient analyte recovery, and/or inhibition of PCR amplification. Consequently, both the detection/quantification and PCR amplification for sequencing may yield false negatives, making it challenging to achieve molecular characterization and variant detection for all samples.

Partial sequencing of 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 CM.7* lineage being the prevalent one. However, mutations characteristic of other lineages, Omicron XBB.1.5*/XBB.1.9*, Omicron XBB.1.16*, and Omicron XBB.2.3*, were also 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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- **Molise:** Michele Colitti (Regione Molise); Carmen Montanaro (ASReM); Giuseppe Aprea, Silvia Scattolini, Daniela D'Angelantonio, Giacomo Migliorati (Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale"); Maria Grazia Cerroni (Arpa Molise);
- **Piemonte:** Bartolomeo Griglio, Renza Berruti, Mauro Cravero, Angela Costa (Regione Piemonte); Manila Bianchi, Lucia Decastelli; Angelo Romano; Clara Tramuta (IZSTO - Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta SC Sicurezza e Qualità degli Alimenti); Elisabetta Carraro, Cristina Pignata (Dipartimento di Scienze della Sanità Pubblica e Pediatriche, Università di Torino), Silvia Bonetta (Dipartimento di Scienze della Vita e Biologia dei Sistemi), Lisa Richiardi (Dipartimento di Scienze della Sanità Pubblica e Pediatriche, Università di Torino);
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- **Sicilia:** Mario Palermo (Regione Sicilia); Carmelo Massimo Maida, Walter Mazzucco (Università degli Studi di Palermo-Dipartimento PROMISE - sezione di Igiene); Simona De Grazia, Giovanni Giammanco (Centro di Riferimento Regionale per la Sorveglianza delle Paralisi Flaccide Acute (PFA) e ambientale della circolazione di poliovirus in Sicilia - AOUP Palermo); Giuseppa Purpari (IZS - Istituto Zooprofilattico Sperimentale della Sicilia); Margherita Ferrante; Antonella Agodi, Martina Barchitta (Università degli Studi di Catania - Dipartimento "G. F. Ingrassia");
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- **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).