Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
21st Report
(Study period: May 8th to May 12th, 2023)

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Main findings:

- During the week of May 8th to May 12th, 2023, a total of 123 wastewater samples were collected from 15 Regions and 2 Autonomous Provinces (AP).
- Mutations characteristic of the omicron variant were identified in 11 of these Regions/APs, while no sequencing data was obtained from the remaining areas.
- Analysis of the sequences obtained by Sanger sequencing showed that 80.6% of the positive samples had amino acid substitutions belonging to the Omicron XBB.1.5*/XBB.1.9* lineages, 9.7% to Omicron XBB*, 6.5% to the Omicron XBB.1.16* and 3.2% to the Omicron CH1.1*.

Introduction

On March 17th, 2021, the European Union Commission issued Recommendation 2021/472, encouraging Member States to establish a systematic surveillance for 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 are monthly sampling campaigns carried out over a short period of time at different locations across Italy. The primary objective of these flash surveys is to gather supplementary information on SARS-CoV-2 variants in the population, to complement the data obtained through clinical surveillance. The aim of this report is to summarize the results from the 21st national Flash Survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from May 8th to May 12th, 2023.

Methodology

During the period from May 8th to May 12th, 2023, the 21st national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out in Italy. The survey involved the collection of 123 wastewater 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]. Samples collected during the survey were processed and the virus 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 extracted from the samples was then sent to ISS for variant detection.

A real-time RT-PCR assay was used to screen for the presence of the Omicron variant[3]. In addition, a long-nested PCR assay covering approximately 1600 base pairs was used, covering amino acid residues 58 to 573 of the spike protein. The assay was used to detect multiple nucleotide changes distinctive of Variants of Concern (VoCs) and Variants of Interest (VoIs) in the

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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] La Rosa G, Iaconelli M, Veneri C, Mancini P, Bonanno Ferraro G, Brandtner D, Lucentini L, Bonadonna L, Rossi M, Grigioni M; SARI network; Suffredini E. The rapid spread of SARS-COV-2 Omicron variant in Italy reflected early through wastewater surveillance. Sci Total Environ. 2022 Sep 1;837:155767. doi: 10.1016/j.scitotenv.2022.155767. Epub 2022 May 6. PMID: 35533857; PMCID: PMC9074219.

spike protein[4]. Following the amplification of target sequences Sanger sequencing was employed for individual samples. For variant classification we adopted a lineage classification based on 'outbreak.info'[5] 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 assignation to sublineages, based solely on the mutations observed in the spike region, not feasible.

Results

Real Time qPCR

Out of the 123 samples collected, a total of 112 (91.1%) 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 1.20E+02 to 1.55E+06 genome copies (g.c.) per liter of sewage. Additionally, 100 out of the 123 samples (81.3%) were found to be positive for the Omicron variant using the RT-qPCR assay, with cycle threshold (CT) values ranging from 29.95 to 39.97.

Sanger Sequencing

Table 1 provides a summary of the results obtained from the real-time PCR assays, long nested PCR, and sequencing methods. A total of 31 samples (25.2%) from 11 Regions/AP were successfully amplified using the long-nested PCR assay. Sanger sequencing confirmed that all the obtained sequences corresponded to the Omicron variant.

The analysis of wastewater samples revealed the presence of different SARS-CoV-2 lineages, as outlined in Tables 1 and 2. The Omicron XBB1.5*/XBB1.9* lineages (which were not distinguishable in the sequenced region) were the most common, detected in 80.6% (25 samples) of the positive samples. In addition, the lineage Omicron XBB* was detected in 9.7% (3 samples), and the lineage Omicron XBB.1.16* in 6.5% (2 samples). The Omicron CH.1.1* lineage was also found in one sample (3.2%).

For ease of understanding, the mutations have been grouped into five 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; S477N; T478K; E484A; F486P; F490S; Q498R; N501Y; Y505H
- **Package B (assigned to the lineage 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; S477N; T478K; E484A; F486S; Q498R; N501Y; Y505H
- **Package C (assigned to the lineage Omicron XBB.1.16*)** = V83A; G142D; DEL 144; H146Q; E180V; Q183E; V213E; G252V; G339H; R346T; L368I; S371F; S373P; S375F;

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[4] G La Rosa, P. Mancini, G. Bonanno Ferraro, C. Veneri, M. Iaconelli, L. Lucentini, L. Bonadonna, S. Brusaferro, D. Brandtner, A. Fasanella, L. Pace, A. Parisi, D. Galante, E. Suffredini. Rapid screening for SARS-CoV-2 variants of concern in clinical and environmental samples using nested RT-PCR assays targeting key mutations of the spike protein, Water Research, 2021, Volume 197, 1 June 2021, 117104. https://doi.org/10.1016/j.watres.2021.117104.
[5] https://outbreak.info/situation-reports, date: 05/10/2023

T376A; D405N; R408S; K417N; N440K; V445P; G446S; N460K; S477N; T478R; E484A; F486P; F490S; Q498R; N501Y; Y505H

- **Package D (assigned to the lineage Omicron XBB*)** = V83A; G142D; DEL 144; H146Q; Q183E; V213E; D215H; G252V; G339H; R346T; L368I; S371F; S373P; S375F; T376A; D405N; R408S; K417N; N440K; V445P; G446S; N460K; S477N; T478K; E484A; F486P; F490S; Q498R; N501Y; Y505H
- **Package E (assigned to the lineage Omicron XBB*)** = V83A; G142D; DEL 144; H146Q; Q183E; V213E; G252V; G339H; R346T; L368I; S371F; S373P; S375F; D405N; R408S; K417N; N440K; V445P; G446S; N460K; S477N; T478K; E484A; F486P; F490S; Q498R; N501Y; Y505H

Package A combined with the 'F456L' mutation, suggests the presence of the Omicron EG.5 sublineage. This sublineage is characterized by having the 'F456L' mutation in approximately 80.5% of its sequences.

Within the XBB* lineage, we identified two distinct mutation packages. Package D, containing the D215H mutation, suggests the presence of the XBB.1.17.1 sublineage, as this sublineage has this specific mutation in 96.2% of its sequences. In addition, package E, which lacks the T376A mutation, suggests the presence of the Omicron XBB.1.33 sublineage.

Lineages XBB.1.5*/XBB.1.9* were detected in 10 regions/A.P. (Abruzzo, Emilia-Romagna, Lazio, Liguria, Lombardia, Molise, Puglia, Sicilia, Veneto and A.P. of Trento). Key mutations of lineage XBB* were observed in 3 regions (Lazio, Liguria and Molise). Characteristic mutations of lineage XBB.1.16* were observed in 2 regions (Friuli Venezia-Giulia and Liguria), and mutations of lineage CH.1.1* were found in only one region (Puglia).

Table 1. PCR and sequencing results

	Sample ID	Region/A.P.	City	WTP	RT-qPCR (c.g./L)	RT-PCR Omicron ID-999 (CT value)	Mutations found by Sanger sequencing (long PCR ID_980)	SARS-CoV-2 lineages
95	20572		Pescara	Villa Carmine	9,31E+02	36.21	Package A	Omicron XBB.1.5*/XBB.1.9*
96	20571		Pescara	Via Raiale	9,31E+02	36.69	Package A	Omicron XBB.1.5*/XBB.1.9*
97	20573	Abruzzo	Chieti	S.Martino	5,88E+02	39.42		
98	20574		L'Aquila	Pile	2,90E+02	38.74		
99	20575		Teramo	Villa Pavone	7,45E+02	35.94		
103	20503	Basilicata	Matera	Pantano	1,08E+04	ND		
104	20502		Potenza	Tiera di Vaglio	2,41E+04	ND		
1	20390		Ferrara	Ferrara - Linea 1	4,16E+04	ND		
2	20391		Ferrara	Ferrara - Linea 2	< LOD	ND		
3	20392		Modena	Carpi	< LOD	ND		
4	20471		Piacenza	Borgoforte	4,70E+04	ND		
5	20472		Parma	Parma ovest	8,53E+04	37.20		
6	20475		Reggio Emilia	Mancasale	8,28E+04	38.43		
115	20516	Emilia-Romagna	Bologna	IDAR	8,30E+04	35.81	Package A	Omicron XBB.1.5*/XBB.1.9*
116	20517		Modena	Naviglio	2,43E+03	ND		
117	20518		Ravenna	Ravenna	1,38E+04	ND		
120	20519		Forlì-Cesena	Forlì	6,25E+03	37.62	Package A	Omicron XBB.1.5*/XBB.1.9*
121	20520		Forlì-Cesena	Cesena	3,58E+04	37.61		
122	20534		Bologna	Imola	3,48E+04	37.18		
124	20537		Ravenna	Ravenna	5,25E+04	ND		
118	20657		Pordenone	Cordenons	2,20E+04	39.54	Package C	Omicron XBB.1.16*
119	20658	Friuli-Venezia-Giulia	Udine	Udine	4,28E+04	38.25		
123	20659		Trieste	Servola	9,85E+03	ND		
7	20351	Lazio	Viterbo	Viterbo - Strada Bagni	3,23E+03	35.15	Package A	Omicron XBB.1.5*/XBB.1.9*

8	20352		Roma	Anzio - Colle Cocchino	1,05E+03	39.77		
9	20354		Latina	Aprilia (Via del Campo)	< LOD	ND		
10	20356		Latina	Latina Loc Latina Est	3,13E+03	34.94		
11	20358		Roma	Pomezia - Via Cincinnato	4,80E+03	34.57	Package E	Omicron XBB*
12	20359		Roma	Velletri (LA CHIUSA-SORBO)	2,02E+03	35.87	Package A	Omicron XBB.1.5*/XBB.1.9*
13	20360		Roma	Guidonia - Ponte Lucano	2,08E+03	35.19	Package A	Omicron XBB.1.5*/XBB.1.9*
14	20375		Roma	Civitavecchia Fiumaretta	< LOD	ND		
15	20442		Genova	Voltri	3,10E+04	36.38	Package A	Omicron XBB.1.5*/XBB.1.9*
16	20443		Genova	Quinto	2,84E+04	37.28		
17	20444		Genova	Rapallo	8,60E+04	33.82		
18	20445		Genova	Sestri P	5,07E+04	35.19	Package C	Omicron XBB.1.16*
19	20446		Genova	Sturla	3,54E+04	35.63	Package A	Omicron XBB.1.5*/XBB.1.9*
20	20447		Savona	Savona	4,30E+04	35.97	Package D[b]	Omicron XBB*[b]
21	20448		Imperia	Imperia	2,83E+04	36.23		
22	20449		Imperia	Sanremo - località Capo Verde	4,50E+04	34.78		
23	20450	Liguria	Savona	Borghetto Santo Spirito	2,63E+04	36.14	Package A	Omicron XBB.1.5*/XBB.1.9*
24	20451		La Spezia	Camisano	1,98E+04	35.74		
25	20452		La Spezia	Silea	2,62E+04	35.21		
26	20453		La Spezia	La Spezia	4,96E+04	34.23		
27	20454		Genova	Darsena	1,28E+04	38.37		
28	20455		Genova	Punta Vagno Genova	2,84E+04	36.31		
29	20456		Genova	Valpolcevera	1,60E+04	36.75	Package A	Omicron XBB.1.5*/XBB.1.9*
87	20441		Genova	Pegli	2,38E+03	ND		
94	20304		Genova	Punta Vagno Genova	1,11E+05	36.59		
30	20402	Lombardia	Sondrio	Sondrio	2,55E+04	37.61		
31	20463		Bergamo	Bergamo	3,33E+04	36.14	Package A	Omicron XBB.1.5*/XBB.1.9*

32	20464		Brescia	Verziano	1,04E+04	36.65		
33	20466		Cremona	Citta di Cremona	1,52E+04	37.50		
88	20403		Milano	Milano Nosedo	1,34E+05	38.46		
89	20404		Milano	Milano San Rocco	1,92E+05	38.70	Package A	Omicron XBB.1.5*/XBB.1.9*
90	20405		Como	Como	< LOD	39.55		
91	20406		Pavia	Pavia	< LOD	38.67		
92	20407		Como - Lecco - Milano - Monza e della Brianza	Monza	< LOD	ND		
93	20408		Pavia	Vigevano	< LOD	ND	Package A + F456L[a]	Omicron XBB.1.5*/XBB.1.9*
34	20480		Pesaro-Urbino	Borgheria	2,15E+04	34.29		
35	20481	Marche	Pesaro-Urbino	Ponte Metauro	2,70E+03	ND		
36	20482		Ancona	Zipa	1,23E+04	35.18		
37	20483		Ancona	Falconara	2,23E+04	34.57		
100	20570		Campobasso - San Pietro	Campobasso	5,39E+02	39.38		
101	20568	Molise	Termoli - località Porto	Termoli	2,28E+03	35.74	Package E[c]	Omicron XBB*[c]
102	20569		Termoli - località Pantano Basso	Termoli	4,84E+02	36.91	Package A	Omicron XBB.1.5*/XBB.1.9*
105	20510		IDA Bolzano	Bolzano	2,91E+04	37.30		
106	20511	P.A. Bolzano	IDA Merano	Bolzano	7,64E+03	38.89		
107	20512		IDA Termeno	Bolzano	7,35E+03	37.75		
38	20329		Trento	Trento nord	6,60E+04	35.46		
39	20330	P.A. Trento	Trento	Trento sud	1,29E+05	34.26		
40	20331		Trento	Rovereto	1,55E+06	29.95	Package A	Omicron XBB.1.5*/XBB.1.9*
41	20317		Torino	Castiglione Torinese	1,63E+03	36.23		
42	20318		Biella	Biella Nord	1,36E+03	36.63		
43	20319		Biella	Biella Sud	5,18E+02	ND		
44	20320	Piemonte	Novara	Novara	1,18E+03	ND		
45	20377		Alessandria	Alessandria	< LOD	ND		
46	20378		Asti	Asti	1,01E+03	38.05		
47	20379		Cuneo	Cuneo	4,88E+03	35.52		

48	20370		Bari	Bari Est	< LOD	ND		
49	20371		Bari	Bari Ovest	9,29E+02	36.19		
50	20380		Brindisi	Brindisi Fiume Grande	3,44E+02	36.60		
51	20381		Lecce	Lecce	3,35E+02	39.23		
52	20382		Taranto	Taranto Bellavista	3,21E+02	36.72		
53	20383		Taranto	Taranto Gennarini	1,28E+03	36.20	Package B	Omicron CH.1.1*
54	20413		Barletta-Andria-Trani	Bisceglie	5,23E+03	35.32	Package A	Omicron XBB.1.5*/XBB.1.9*
55	20414	Puglia	Barletta-Andria-Trani	Andria	1,97E+03	33.28	Package A	Omicron XBB.1.5*/XBB.1.9*
56	20415		Barletta-Andria-Trani	Barletta	2,21E+03	39.39		
57	20416		Barletta-Andria-Trani	Trani	1,07E+03	36.60		
58	20417		Foggia	Cerignola	1,96E+03	39.32		
59	20418		Foggia	Manfredonia	1,49E+03	39.14		
60	20419		Foggia	Foggia	1,89E+03	35.16	Package A	Omicron XBB.1.5*/XBB.1.9*
61	20468		Bari	Molfetta	1,21E+03	35.14		
62	20478		Bari	Bitonto	7,79E+03	33.52	Package A	Omicron XBB.1.5*/XBB.1.9*
63	20479		Bari	Altamura	5,99E+02	39.32		
64	20353		Trapani	Trapani	2,52E+03	37.93		
65	20355		Trapani	Mazara del Vallo	1,32E+03	39.03		
66	20357		Trapani	Marsala	7,79E+03	35.07		
67	20428		Agrigento	Agrigento	2,02E+04	38.06		
68	20429		Enna	Enna	7,63E+04	35.23	Package A	Omicron XBB.1.5*/XBB.1.9*
69	20430		Palermo	Bagheria	1,43E+04	39.95		
70	20432	Sicilia	Palermo	Acqua dei Corsari	2,11E+04	39.31		
71	20434		Palermo	Fondo Verde	1,69E+04	38.33		
72	20435		Caltanissetta	Caltanissetta e San Cataldo	2,79E+04	37.33		
108	20484		Catania	Pantano d'Arci	2,01E+04	38.77	Package A	Omicron XBB.1.5*/XBB.1.9*
109	20485		Catania	Giarre	1,47E+04	37.02		
110	20486		Siracusa	Siracusa	2,28E+04	38.15		

111	20458		Vittoria	Ragusa	1,00E+03	39.68		
112	20460		Gela Macchitella	Caltanissetta	4,25E+02	39.60		
113	20461		Mili Marina	Mili Marina	1,25E+03	37.95		
114	20462		Mili Marina	Mili Marina	6,75E+02	39.00	Package A	Omicron XBB.1.5*/XBB.1.9*
73	20401	Umbria	Perugia	Perugia - Pian della Genna	1,19E+04	39.04		
74	20490	Valle d'Aosta	Aosta	La Salle	1,20E+02	ND		
75	20491		Aosta	Brissogne	1,20E+02	39.61		
76	20325		Padova	Padova Ca' Nordio - centro storico	4,10E+04	37.92		
77	20326		Padova	Padova Ca' Nordio - zip	3,94E+04	ND		
78	20327		Padova	Padova Guizza	5,15E+04	ND	Package A	Omicron XBB.1.5*/XBB.1.9*
79	20328		Padova	Abano Terme	3,12E+03	39.97		
80	20372	Veneto	Treviso	Treviso	2,10E+03	36.43		
81	20373		Vicenza	Vicenza Casale	1,83E+03	37.02		
82	20374		Venezia	Venezia Fusina	3,02E+03	34.49		
83	20396		Verona	Verona_collettore 1M	2,20E+03	35.61		
84	20397		Verona	Verona_collettore 3M	6,06E+03	34.59	Package A	Omicron XBB.1.5*/XBB.1.9*
85	20398		Verona	Verona_collettore 8M	< LOD	35.67		

[a] 'Package A' combined with the '**F456L**' mutation, suggests the presence of the Omicron EG.5 sublineage. This sublineage is characterized by having the 'F456L' mutation in approximately 80.5% of its sequences. The Omicron XBB.1.5* and Omicron XBB.1.9* lineages may also carry the F456L mutation, but at lower rates of 3.0% and 26.6%, respectively. (https://outbreak.info/situation-reports, date: 05/10/2023);

[b] Package D, containing the D215H mutation, suggests the presence of the XBB.1.17.1 sublineage, as this sublineage has this specific mutation in 96.2% of its sequences.

[c] Package E, which lacks the T376A mutation, suggests the presence of the Omicron XBB.1.33 sublineage.

ND: not detected

Table 2. Sanger sequencing results

ID SAMPLES	V83A	G142D	DEL144	H146Q/H146K	K147E	W152R	F157L	E180V	Q183E	I210V	V213G	V213E	D215H	G252V	G257S	G339H	R346T	L368I	S371F	S373P	S375F	T376A	D405N	R408S	K417N	N440K	K444T	V445P	G446S	F456L	L452R	N460K	S477N	T478K	T478R	E484A	F486S	F486P	F490S	Q498R	N501Y	Y505H	PACKAGES	LINEAGES
7, 12, 13, 15, 19, 23, 29, 31, 40, 54, 55, 60, 62, 68, 78, 84, 89, 95, 96, 102, 108, 114, 115, 120	■	■	■	■					■			■		■		■	■	■	■	■	■	■	■	■	■	■		■	■			■	■	■		■		■	■	■	■	■	**Package A**	**Omicron XBB.1.5*/1.9***
93	■	■	■	■					■			■		■		■	■	■	■	■	■	■	■	■	■	■		■	■	■		■	■	■		■		■	■	■	■	■	**Package A + F456L**	
53		■			■	■	■			■		■			■	■				■	■	■	■	■	■		■	■				■	■	■	■	■		■	■	■	■	■	**Package B**	**Omicron CH.1.1***
18, 118	■	■	■	■				■	■			■		■		■	■	■	■	■	■	■	■	■	■	■		■	■			■	■		■	■		■	■	■	■	■	**Package C**	**Omicron XBB.1.16***
20	■	■	■	■					■			■	■			■	■	■	■	■	■	■	■	■	■	■		■	■			■	■	■		■		■	■	■	■	■	**Package D**	**Omicron XBB***
11, 101	■	■	■	■					■			■		■		■	■	■	■	■		■	■	■	■	■		■	■			■	■	■		■		■	■	■	■	■	**Package E**	

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. As a result, both the detection/quantification and PCR amplification for sequencing may yield false negatives, making it difficult 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 gather supplementary information on SARS-CoV-2 variants in the population, complementing data obtained through clinical surveillance. The findings from this survey indicate that the Omicron variant is the sole of SARS-CoV-2 variant in Italy, with the lineages XBB.1.5*/XBB.1.9* being the prevalent. However, mutations characteristic of other lineages, XBB*, XBB.1.16* and CH.1.1* were also detected.

Sequencing SARS-CoV-2 in wastewater samples provides valuable additional insights alongside the sequencing of clinical cases. This approach enables a more comprehensive and accurate understanding of the circulating variants in the country, contributing to a better characterization of the virus's spread and evolution.