Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
44th Report
(Study period: March 31st to April 4th, 2025)

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Main findings:

- During the week of 31 March to 4 April 2025, a total of 107 wastewater samples were collected from 16 Regions and two Autonomous Provinces (A.P.s).
- Mutations characteristic of the Omicron variant were identified in three regions, while sequencing data were not available from the remaining regions/AP.
- Sanger sequencing analysis showed that all positive samples carried amino acid substitutions typical of the Omicron JN.1* lineage, including mutations associated with the LP.8.1*, LB.1.2.1, XEC*, XDY.1, KP.3*/JN.1.16* and KP.2*/LP* sublineages.

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 44th national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from 31 March to 4 April 2025.

Methodology

The 44th national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out in Italy from 31 March to 4 April 2025. The survey involved the collection of 107 wastewater samples from 103 wastewater treatment plants (WTPs) located in 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, a long-nested PCR assay was employed, covering approximately 1330 base pairs and spanning from amino acid residues 34 to 475 of the spike protein (PCR ID 1033/1034). After the target sequences were amplifyed, individual samples were subjected to Sanger sequencing.
For variant classification, a lineage classification based on 'outbreak.info'[3] was adopted instead of specifying sublineages. This choice was made because numerous sublineages 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 unfeasible.

Results

Real Time qPCR

Real-time PCR was performed on 76 of the 107 samples. Out of the 76 samples analysed, a total of 41 (53.9%) 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 9.77E + 01 to 5.20E + 04 genome copies (g.c.) per liter of sewage.

Sanger Sequencing

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

Table 1 summarises the results of the long-nested PCR assay and sequencing methods. A total of 17/107 samples (15.9%) from 3 Regions were successfully amplified using the long-nested PCR assay described above.

Analysis of the wastewater samples confirmed the exclusive presence of the Omicron JN.1* lineage, as shown in Tables 1 and 2. This lineage was detected in three regions/A.P.: Friuli Venezia Giulia, Liguria and Veneto.

Within this lineage, 23.5% of the samples (4/17 samples) showed mutation associated with the LP.8.1* sublineage, 17.6% (3/17 samples) with the LB.1.2.1 sublineage, 17.6% (3/17 samples) with the XEC* sublineage, 17.6% (3/17 samples) with the XDY.1 sublineage, 11.8% (2/17 samples) with the KP.3*/JN.1.16* sublineage and 11.8% (2/17 samples) with the KP.2*/LP*.

The observed mutations are grouped into a single panel, referred to as a "mutation package", listed below:

Package A (assigned to the Omicron JN.1*) = DEL69/70, V127F, G142D, DEL144, F157S, R158G, DEL211/212, V213G, L216F, H245N, A264D, I332V, G339H, K356T, S371F, S373P, S375F, T376A, R403K, D405N, R408S, K417N, N440K, V445H, G446S, N450D, L452W, L455S, N460K

Table 1. PCR and sequencing results

ID ISS	ID SARI	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)
74	29335	Abruzzo	Pescara	Villa Carmine	<LOD		
75	29337		Pescara	Via Raiale	<LOD		
76	29336		Chieti	S. Martino	<LOD		
77	29338		L'Aquila	Pile	<LOD		
78	29334		Teramo	Villa Pavone	<LOD		
88	29344	Basilicata	Potenza	Tiera di Vaglio	<LOD		
89	29346		Matera	Pantano	<LOD		
127	29479	Campania	Napoli	Napoli OVEST - Ingresso Principale	<LOD		
128	29480		Napoli	Napoli OVEST - ex ingresso Camaldoli	4,55E+02		
129	29478		Napoli	Napoli EST	<LOD		
67	29400	Emilia-Romagna	Modena	Carpi	<LOD		
68	29398		Ferrara	Ferrara - Linea 1	<LOD		
69	29399		Ferrara	Ferrara - Linea 2	<LOD		
90	29352		Piacenza	Borgoforte	<LOD		
91	29353		Parma	Parma ovest	<LOD		
97	29320		Modena	Naviglio	<LOD		
98	29321		Bologna	Imola	1,54E+03		
99	29322		Forlì-Cesena	Forlì	7,46E+03		
100	29324		Bologna	IDAR	2,10E+03		
101	29325		Rimini - Forlì-Cesena	S. Giustina	6,82E+02		
102	29326		Ravenna	Faenza	<LOD		
48	29307	Friuli-Venezia Giulia	Udine	Udine	4,90E+03	Package A + F456L[a]	Omicron JN.1*
49	29308		Trieste	Servola	2,50E+03		
50	29309		Pordenone	Cordenons	5,20E+04	Package A + F59S + F456L[b] + K182R	Omicron JN.1*
47	29240	Lazio	Roma	Civitavecchia Fiumaretta	<LOD		
60	29361		Latina	Latina Loc Latina Est	NA		

61	29362		Latina	Aprilia (Via del Campo)	NA		
62	29363		Roma	Anzio - Colle Cocchino	NA		
63	29364		Roma	Pomezia - Via Cincinnato	NA		
64	29365		Roma	Velletri (LA CHIUSA-SORBO)	NA		
65	29366		Roma	Guidonia - Ponte Lucano	NA		
66	29377		Viterbo	Viterbo - Strada Bagni	NA		
10	29275	Liguria	Genova	Pegli	<LOD	Package A + F186L + R190S + R346T + V445R + F456L[c] + N185B	Omicron JN.1*
11	29276		Genova	Voltri	8,88E+03		
12	29277		Genova	Sestri P	6,55E+03	Package A + Q183H + R190T + A435S + F456L[d]	Omicron JN.1*
13	29278		Genova	Valpolcevera	6,45E+03	Package A + Q183H + R190T + R346T + F456L[e] + A435S	Omicron JN.1*
14	29279		Genova	Rapallo	<LOD	Package A + Q183H + R190T + A435S + F456L[d]	Omicron JN.1*
15	29281		Genova	Quinto	3,61E+03		
16	29282		Genova	Punta Vagno Genova	3,23E+03	Package A + F186L + R190S + R346T + V445R + F456L[c] + A67P	Omicron JN.1*
17	29283		Genova	Darsena	<LOD		
18	29284		Genova	Sturla	6,92E+03		
19	29286		La Spezia	La Spezia	<LOD		
45	29280		Savona	Borghetto Santo Spirito	5,06E+03	Package A + R346T + F456L[f]	Omicron JN.1*
46	29285		Savona	Savona	3,67E+03	Package A + F59S + F456L[b] +R78S	Omicron JN.1*
92	29354		Genova	Recco	<LOD		
70	NA	Lombardia	Brescia	Verziano	NA		
71	NA		Bergamo	Bergamo	NA		
72	NA		Monza	Monza	NA		
73	NA		Cremona	Cremona	NA		
110	NA		Sondrio	Sondrio	NA		
111	NA		Monza	Monza	NA		
112	NA		Lecco	Lecco	NA		
113	NA		Milano - Monza e della Brianza	Peschiera Borromeo	NA		
114	NA		Varese	Varese	NA		
115	NA		Milano	Milano Nosedo	NA		

116	NA		Milano	Milano S. Rocco	NA
117	NA		Como	Como	NA
118	NA		Pavia	Pavia	NA
119	NA		Lodi	Lodi	NA
54	29345	Marche	Pesaro-Urbino	Borgheria	6,23E+02
55	29347		Pesaro-Urbino	Ponte Metauro	<LOD
56	29348		Ancona	Zipa	<LOD
57	29349		Ancona	Falconara	<LOD
58	29350		Ascoli Piceno	Marino del Tronto	<LOD
59	29351		Fermo	Salvano	<LOD
79	29341	Molise	Campobasso	Termoli - località Pantano Basso	<LOD
80	29340		Campobasso	Termoli - località Porto	<LOD
81	29339		Campobasso	Campobasso - San Pietro	<LOD
85	29392	A.P. Bolzano	Bolzano	IDA Bolzano	2,16E+03
86	29393		Bolzano	IDA Merano	4,48E+03
87	29394		Bolzano	IDA Termeno	1,15E+02
51	29295	A.P. Trento	Trento	Trento nord	2,47E+04
52	29296		Trento	Trento sud	3,15E+04
53	29297		Trento	Rovereto	2,90E+04
120	29217	Piemonte	Torino	Castiglione Torinese	NA
121	29218		Biella	Biella Nord	NA
122	29219		Biella	Biella Sud	NA
123	29220		Novara	Novara	NA
124	29257		Alessandria	Alessandria	1,07E+04
125	29258		Asti	Asti	<LOD
126	29259		Cuneo	Cuneo	5,55E+03
93	29230	Puglia	Bari	Bari Est	<LOD
94	29231		Bari	Bari Ovest	<LOD
95	29232		Taranto	Taranto Bellavista	9,77E+01
96	29233		Taranto	Taranto Gennarini	<LOD

82	29327	Sicilia	Catania	Pantano d'Arci	2,37E+03		
83	29328		Catania	Giarre	<LOD		
84	29329		Siracusa	Siracusa	4,96E+03		
103	29383		Palermo	Acqua dei Corsari	9,63E+02		
104	29384		Palermo	Fondo Verde	1,03E+03		
105	29385		Caltanissetta	Caltanissetta e San Cataldo	1,21E+03		
106	29387		Agrigento	Agrigento	4,48E+03		
107	29234		Trapani	Trapani	4,30E+02		
108	29235		Trapani	Mazara del Vallo	6,30E+02		
109	29236		Trapani	Marsala	7,50E+02		
22	29291	Toscana	Pisa	Pisa Nord - S. Jacopo	NA		
23	29255	Umbria	Perugia	Perugia - Pian della Genna	NA		
24	29237	Veneto	Treviso	Treviso	5,34E+03		
25	29238		Venezia	Venezia Fusina	6,91E+03	Package A + Q183H + R190T + R346T + F456L[e]	Omicron JN.1*
26	29239		Vicenza	Vicenza Casale	2,79E+03		
27	29288		Verona	Verona_collettore 1M	9,02E+03	Package A + F456L[a]	Omicron JN.1*
28	29289		Verona	Verona_collettore 3M	1,32E+04	Package A + Q183H + R190T + R346T + F456L[e]	Omicron JN.1*
29	29290		Verona	Verona_collettore 8M	1,34E+04	Package A + Q183H + R190T + A435S + F456L[d]	Omicron JN.1*
30	29330		Padova	Padova Ca' Nordio - centro storico	NA	Package A + R346T + F456L[f]	Omicron JN.1*
31	29331		Padova	Padova Ca' Nordio - zip	NA	Package A + F59S + F456L[b]	Omicron JN.1*
32	29332		Padova	Padova Guizza	NA	Package A + F186L + R190S + R346T + V445R + F456L[c] + A67P	Omicron JN.1*
33	29333		Padova	Abano Terme	NA	Package A + F186L + R190S + R346T + V445R + F456L[c] + A67P	Omicron JN.1*

NA= Not available

[a] The key mutations of Omicron JN.1* in association with F456L may indicate the presence of the Omicron KP.3* or JN.1.16* sublineages.

[b] The key mutations of Omicron JN.1* in association with F59S and F456L may indicate the presence of the Omicron XEC* sublineage.

[C] The key mutations of Omicron JN.1* in association with F186L, R190S, R346T, V445R and F456L may indicate the presence of the Omicron LP.8.1* sublineages.

[d] The key mutations of Omicron JN.1* in association with Q183H, R190T, A435S and F456L may indicate the presence of the Omicron XDY.1 sublineages.

[e] The key mutations of Omicron JN.1* in association with Q183H, R190T, R346T and F456L may indicate the presence of the Omicron LB.1.2.1 sublineages.

[f] The key mutations of Omicron JN.1* in association with R346T and F456L may indicate the presence of the Omicron KP.2*/LP* sublineages.

Table 2. Sanger sequencing results

ID SAMPLES	F59S	DEL69/70	V127F	G142D	DEL 144	F157S	R158G	Q183H	F186L	R190S	R190T	DEL211/212	V213G	L216F	H245N	A264D	I332V	G339H	R346T	K356T	S371F	S373P	S375F	T376A	R403K	D405N	R408S	K417N	A435S	N440K	V445R	V445H	G446S	N450D	L452W	L455S	F456L	N460K	VARIANTS
48, 27		■	■	■	■	■	■					■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■		■		■	■	■	■	■	■	■	**Package A (Omicron JN.1* + F456L)**
31, 46, 50	■	■	■	■	■	■	■					■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■		■		■	■	■	■	■	■	■	**Package A (Omicron JN.1* + F59S + F456L)**
10, 16, 32, 33		■	■	■	■	■	■		■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■		■	■	■	■	■	■	**Package A (Omicron JN.1* + F186L + R190S + R346T + V445R + F456L)**
12, 14, 29		■	■	■	■	■	■	■			■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	**Package A (Omicron JN.1* + Q183H + R190T + A435S + F456L)**
13, 25, 28		■	■	■	■	■	■	■			■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■		■	■	■	■	■	■	■	**Package A (Omicron JN.1* + Q183H + R190T + R346T + F456L)**
45, 30		■	■	■	■	■	■					■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■		■	■	■	■	■	■	■	**Package A (Omicron JN.1* + R346T + F456L)**

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 molecular characterization and variant detection achievement difficult 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 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 findings from this survey confirm that the Omicron JN.1 lineage is the only SARS-CoV-2 variant detected in wastewater in Italy, with mutations associated with various sublineages. 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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