Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
31st Report
(Study period: March 4th to March 8th, 2024)

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Main findings:

- During the week of 4 March to 8 March 2024, a total of 97 wastewater samples were collected from 16 Regions and 2 Autonomous Province (A.P.).
- Mutations characteristic of the Omicron variant were identified in 8 of these Regions/AP, while no sequencing data were collected from the remaining areas.
- Analysis of the sequences obtained by Sanger sequencing showed that 100% of the positive samples displayed amino acid substitutions that are typical of the Omicron JN.1* 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 31st national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from 4 to 8 March 2024.

Methodology

The 31st national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out in Italy from 4 to 8 March 2024. The survey involved the collection of 100 wastewater samples from 96 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]. 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 region, making a reliable assignation to sublineages, based solely on the mutations observed in the spike region, not feasible.

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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

Results

Real Time qPCR

Of the 100 samples analysed, a total of 48 (48%) tested positive for SARS-CoV-2 using the real-time RT-qPCR method employed for environmental surveillance (Table 1). The detected viral concentrations in these samples varied, ranging from 2.08E + 02 to 1.55E + 05 genome copies (g.c.) per liter of sewage.

Sanger Sequencing

Table 1 summarises the results of the long-nested PCR assay and sequencing methods. A total of 14 samples (14%) from 8 Regions/AP were successfully amplified using the long-nested PCR assay described above. Sequences from 14 of these samples were obtained by Sanger sequencing.
The analysis of the wastewater samples revealed the presence of one SARS-CoV-2 lineage, as shown in Tables 1 and 2. The Omicron JN.1* lineage was detected in 100% of positive samples.
For ease of understanding, the mutations have been grouped into panels or "mutation packages". These are 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

The Omicron JN.1* was detected in 8 regions/A.P.: Abruzzo, Emilia-Romagna, Liguria, Lombardia, Molise, Puglia, Sicilia and A.P. Bolzano.

Table 1. PCR and sequencing results

ID ISS	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)
74	24928	Abruzzo	Pescara	Via Raiale	2,08E+02	Package A (partial sequence, from V127F to S373P)[a]	Omicron JN.1*
75	24923		Pescara	Villa Carmine	<LOD		
76	24924		Chieti	S. Martino	<LOD		
77	24929		L'Aquila	Pile	<LOD		
78	24930		Teramo	Villa Pavone	<LOD		
82	24915	Basilicata	Potenza	Tiera di Vaglio	2,25E+03		
83	24916		Matera	Pantano	<LOD		
95	24976	Campania	Napoli	Napoli EST	<LOD		
96	24977		Napoli	Napoli OVEST - Ingresso Principale	<LOD		
97	24978		Napoli	Napoli OVEST - ex ingresso Camaldoli	5,29E+03		
1	24853	Emilia-Romagna	Piacenza	Borgoforte	2,67E+04	Package A (partial sequence, from G142D to S373P)[a]	Omicron JN.1*
2	24857		Ferrara	Ferrara - Linea 1	<LOD		
3	24858		Ferrara	Ferrara - Linea 2	<LOD		
4	24859		Modena	Carpi	<LOD		
5	24854		Parma	Parma ovest	<LOD		
6	24855		Reggio Emilia	Mancasale	5,38E+03		
84	24979		Forlì-Cesena	Forlì	3,13E+03	Package A + R346T	Omicron JN.1*
85	24980		Forlì-Cesena	Cesena	1,05E+03	Package A (partial sequence, from V127F to S373P)[a]	Omicron JN.1*
86	24981		Bologna	IDAR	7,50E+02		

87	24989		Ravenna - Forlì-Cesena	Ravenna	2,75E+02	Package A (partial sequence, from V127F to S373P)[a]	Omicron JN.1*
88	24990		Ravenna	Faenza	7,25E+02		
89	24991		Bologna	Imola	9,83E+04		
90	24992		Modena	Naviglio	7,50E+03		
91	24993		Rimini - Forlì-Cesena	S. Giustina	8,25E+02		
98	24925	Friuli-Venezia Giulia	Pordenone	Cordenons	4,36E+03		
99	24926		Udine	Udine	2,24E+03		
100	24927		Trieste	Servola	4,33E+03		
7	24841	Lazio	Roma	Civitavecchia Fiumaretta	<LOD		
8	24877	Liguria	Imperia	Imperia	<LOD		
9	24856		Genova	Recco	<LOD		
10	24864		Genova	Pegli	<LOD		
11	24865		Genova	Voltri	6,83E+03	Package A (partial sequence, from V127F to S373P)[a]	Omicron JN.1*
12	24866		Genova	Quinto	<LOD		
13	24867		Genova	Rapallo	2,32E+03		
14	24868		Genova	Sestri P	<LOD		
15	24869		Genova	Sturla	<LOD	Package A (partial sequence, from V127F to S373P)[a]	Omicron JN.1*
16	24870		Genova	Darsena	2,28E+03		
17	24871		Genova	Punta Vagno Genova	2,42E+03		
18	24873		La Spezia	La Spezia	2,55E+03		
19	24874		Savona	Borghetto Santo Spirito	<LOD		
20	24876		Imperia	Sanremo - località Capo Verde	3,53E+03		
21	24872		Genova	Valpolcevera	<LOD		
22	24875		Savona	Savona	<LOD		
23	24889	Lombardia	Bergamo	Bergamo	<LOD		
24	24890		Cremona	Città di Cremona	<LOD		

25	24893		Brescia	Verziano	<LOD		
62	24883		Pavia	Vigevano	<LOD		
63	24881		Pavia	Pavia	<LOD		
64	24878		Milano	Milano Nosedo	<LOD		
65	24879		Milano	Milano San Rocco	1,55E+05		
66	25273		Sondrio	Sondrio	1,22E+04		
67	24882		Como - Lecco - Milano - Monza e della Brianza	Monza	<LOD	Package A (partial sequence, from G142D to S373P)[a]	Omicron JN.1*
68	24880		Como	Como	<LOD		
26	24904	Marche	Pesaro-Urbino	Borgheria	1,15E+03		
27	24905		Pesaro-Urbino	Ponte Metauro	<LOD		
28	24907		Ancona	Zipa	<LOD		
29	24908		Ancona	Falconara	<LOD		
79	24932	Molise	Campobasso	Termoli - località Pantano Basso	<LOD	Package A (partial sequence, from V127F to S373P)[a]	Omicron JN.1*
80	24931		Campobasso	Termoli - località Porto	<LOD	Package A (partial sequence, from V127F to S373P)[a]	Omicron JN.1*
81	24933		Campobasso	Campobasso - San Pietro	<LOD	Package A (partial sequence, from V127F to S373P)[a]	Omicron JN.1*
71	24934	P.A. Bolzano	Bolzano	IDA Bolzano	3,76E+03	Package A (partial sequence, from V127F to S373P)[a]	Omicron JN.1*
72	24935		Bolzano	IDA Merano	1,48E+03		
73	24936		Bolzano	IDA Termeno	3,43E+03		
30	24817	P.A. Trento	Trento	Trento nord	6,44E+03		
31	24818		Trento	Trento sud	2,47E+03		
32	24843		Trento	Rovereto	3,46E+03		

33	24830	Piemonte	Alessandria	Alessandria	<LOD		
34	24831		Asti	Asti	<LOD		
35	24832		Cuneo	Cuneo	<LOD		
36	24806		Torino	Castiglione Torinese	3,50E+03		
37	24807		Biella	Biella Nord	<LOD		
38	24808		Biella	Biella Sud	<LOD		
39	24809		Novara	Novara	<LOD		
40	24819	Puglia	Bari	Bari Est	<LOD		
41	24820		Bari	Bari Ovest	<LOD	Package A (partial sequence, from G142D to S373P)[a]	Omicron JN.1*
42	24821		Taranto	Taranto Bellavista	<LOD		
43	24822		Taranto	Taranto Gennarini	4,12E+02		
44	24823	Sicilia	Trapani	Trapani	1,75E+03		
45	24824		Trapani	Mazara del Vallo	2,03E+03		
46	24825		Trapani	Marsala	1,36E+03		
47	24888		Caltanissetta	Gela Macchitella	8,50E+02		
48	24898		Palermo	Fondo Verde	1,68E+03		
49	24899		Caltanissetta	Caltanissetta e San Cataldo	3,91E+03		
50	24900		Palermo	Bagheria	1,54E+03		
92	24901		Catania	Pantano d'Arci	<LOD		
93	24902		Catania	Giarre	4,14E+03	Package A (partial sequence, from G142D to S373P)[a]	Omicron JN.1*
94	24903		Siracusa	Siracusa	1,66E+03		
69	24906	Toscana	Pisa	Pisa Nord - S. Jacopo	<LOD		
70	24909		Lucca	Pontetetto	<LOD		
51	24842	Umbria	Perugia	Perugia - Pian della Genna	5,64E+03		
52	24861	Veneto	Verona	Verona_collettore 1M	8,90E+03		
53	24862		Verona	Verona_collettore 3M	1,02E+04		
54	24863		Verona	Verona_collettore 8M	4,06E+03		
55	24813		Padova	Padova Ca' Nordio - centro storico	4,50E+04		

56	24814		Padova	Padova Ca' Nordio - zip	9,82E+03
57	24815		Padova	Padova Guizza	2,01E+04
58	24816		Padova	Abano Terme	8,64E+03
59	24826		Treviso	Treviso	6,82E+02
60	24827		Vicenza	Vicenza Casale	<LOD
61	24828		Venezia	Venezia Fusina	7,53E+02

[a] 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	DEL69/70	V127F	G142D	DEL 144	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	VARIANTS
1, 11, 15, 41, 67, 74, 71, 79, 80, 81, 84, 95, 87, 93	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	**Package A (Omicron JN.1*)**

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 results of this survey indicate that the Omicron variant is the sole SARS-CoV-2 variant in Italy, with the Omicron JN.1* lineage currently circulating. 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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