Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
29th Report
(Study period: January 8th to January 12th, 2024)

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Main findings:

- During the week of 8 January to 12 January 2024, a total of 91 wastewater samples were collected from 14 Regions and 2 Autonomous Province (A.P.).
- Mutations characteristic of the Omicron variant were identified in 12 of these Regions/AP, while no sequencing data were collected from the remaining areas.
- Analysis of the sequences obtained by Sanger sequencing revealed that 79% of the positive samples displayed amino acid substitutions typical of the Omicron JN.1* lineage and the 21% percentage exhibited substitutions associated with the XBB.1.5/1.9* lineages.

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 29th national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from 8 to 12 January 2024.

Methodology

The 29th national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out from 8 to 12 January 2024. The survey entailed 91 sewage samples from 89 wastewater treatment plants (WTPs) located across 14 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 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 were delivered to ISS for variant detection.

For sequencing purposes, we employed a long-nested PCR assay covering approximately 1330 base pairs, spanning from amino acid residues 34 to 475 of the spike protein (PCR ID 1033/1034). Following 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]

Results

Real Time qPCR

Out of the 86 samples analysed, a total of 83 (96.5%) tested positive for SARS-CoV-2 using the real-time RT-qPCR method employed for environmental surveillance (Table 1). The viral

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

concentrations detected in these samples varied, ranging from 2.50E + 02 to 2.84E + 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 35 samples (38.5%) from 12 Regions/AP were successfully amplified using the long-nested PCR assay described above. Among these, high-quality sequences were obtained from 33 samples by Sanger sequencing, while 2 samples yielded only partial sequences due to high signal noise.
Analysis of the wastewater samples revealed the presence of five SARS-CoV-2 lineages, as shown in Tables 1 and 2. Among these, the Omicron JN.1* lineage emerged as the most prevalent, being detected in 78.8% of the positive samples (26 samples). Following closely were the Omicron XBB.1.5/1.9* lineages (which are not distinguishable in the sequenced region), detected in 21.2% (7 samples).
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*)** [4] = 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 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

Mutations of the Omicron XBB.1.5*/XBB.1.9* lineages were identified in 6 regions: Emilia-Romagna, Liguria, Lombardia, Molise, Sicilia and Veneto. Instead, mutations of the Omicron JN.1* lineage were observed in 11 regions/A.P.: Abruzzo, Emilia-Romagna, Liguria, Marche, Molise, Piemonte, Puglia, Sicilia, Toscana, Veneto and A.P. Trento.

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[4] 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: 26/01/2024).

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	24153		Pescara	Via Raiale	6,92E+02	Package C	Omicron JN.1*
2	24154		Pescara	Villa Carmine	<LOD		
3	24155	Abruzzo	Chieti	S. Martino	3,34E+02		
4	24156		Teramo	Villa Pavone	1,43E+03		
5	24157		L'Aquila	Pile	7,21E+02		
6	24185	Basilicata	Potenza	Tiera di Vaglio	2,46E+04		
7	24186		Matera	Pantano	1,91E+05		
14	24223		Bologna	IDAR	3,65E+03		
15	24224		Forlì-Cesena	Cesena	4,55E+03	Package C	Omicron JN.1*
16	24225		Forlì-Cesena	Forlì	6,93E+03		
17*	24226		Ravenna	Faenza	4,78E+03	Package B + H146K+ F157L + L452R[b]	Omicron XBB.1.5*/XBB.1.9*
18	24227		Bologna	Imola	9,75E+03		
19	24228	Emilia-Romagna	Modena	Naviglio	3,18E+04		
20	24229		Ravenna - Forlì-Cesena	Ravenna	2,58E+04	Package C	Omicron JN.1*
21	24230		Rimini - Forlì-Cesena	S. Giustina	6,58E+04		
103	24077		Modena	Carpi	3,32E+04		
98	24075		Ferrara	Ferrara - Linea 1	1,16E+04		
99	24076		Ferrara	Ferrara - Linea 2	<LOD		
22	23956	Lazio	Roma	Civitavecchia Fiumaretta	2,50E+02		
23*	24090		Savona	Savona	3,20E+04	Package B[b]	Omicron XBB.1.5*/XBB.1.9*
24	24091	Liguria	Savona	Borghetto Santo Spirito	9,83E+04		
25	24092		Imperia	Imperia	1,55E+04		
26	24093		La Spezia	La Spezia	1,06E+04		

27	24094		Genova	Pegli	3,89E+03	Package C + P330S	Omicron JN.1*
28	24095		Genova	Voltri	7,17E+04	Package C	Omicron JN.1*
29	24096		Genova	Quinto	3,46E+04	Package C	Omicron JN.1*
30	24097		Genova	Rapallo	1,10E+04		
31	24098		Genova	Sestri P	1,13E+04		
32	24099		Genova	Sturla	1,31E+04		
33	24100		Genova	Darsena	9,17E+03		
34	24101		Genova	Punta Vagno Genova	2,39E+04		
35	24102		Genova	Valpolcevera	8,34E+03		
37	24084		Milano	Milano Nosedo	NA		
38	24085		Milano	Milano San Rocco	NA		
39	24086		Como	Como	NA	Package C	Omicron JN.1*
40	24087		Pavia	Pavia	NA		
41	24088	Lombardia	Como - Lecco - Milano - Monza e della Brianza	Monza	NA		
43	24117		Bergamo	Bergamo	3,22E+04	Package B + L455F[b]	Omicron XBB.1.5*/XBB.1.9*
44	24118		Brescia	Verziano	2,61E+04		
45	24120		Cremona	Citta di Cremona	2,63E+04	Package B + L455F[b]	Omicron XBB.1.5*/XBB.1.9*
91	24411		Sondrio	Sondrio	9,09E+03		
46	24132		Pesaro-Urbino	Borgheria	2,43E+04		
47	24133		Pesaro-Urbino	Ponte Metauro	1,34E+04		
48	24134	Marche	Ancona	Zipa	4,58E+04	Package C + I210V	Omicron JN.1*
49	24135		Ancona	Falconara	1,89E+04		
50	24158		Campobasso	Campobasso - San Pietro	1,27E+03	Package C	Omicron JN.1*
51	24159		Campobasso	Termoli - località Porto	3,62E+03	Package C	Omicron JN.1*
52	24160	Molise	Campobasso	Termoli - località Pantano Basso	2,49E+03	Package B (Partial, from Q52H to G142D, from V213E to N460K)[c]	Omicron XBB.1.5*/XBB.1.9*
53	24188	P.A. Bolzano	Bolzano	IDA Bolzano	1,55E+04		
54	24189		Bolzano	IDA Merano	4,64E+04		

55	24190		Bolzano	IDA Termeno	7,27E+04	
56	24033		Trento	Trento nord	2,14E+05	Package C	Omicron JN.1*
57	24034	P.A. Trento	Trento	Trento sud	2,71E+05	Package C	Omicron JN.1*
58	24035		Trento	Rovereto	2,84E+05	
59	24021		Torino	Castiglione Torinese	8,83E+04	
60	24022		Biella	Biella Nord	2,80E+04	
61	24023		Biella	Biella Sud	5,33E+04	
62	24024	Piemonte	Novara	Novara	5,43E+04	
63	24072		Alessandria	Alessandria	1,35E+04	
64	24073		Asti	Asti	5,10E+03	
65	24074		Cuneo	Cuneo	5,60E+03	Package C	Omicron JN.1*
66	24029		Bari	Bari Est	5,04E+03	ME	
67	24030		Bari	Bari Ovest	9,73E+03	ME	
68	24031	Puglia	Taranto	Taranto Bellavista	6,04E+03	Package C	Omicron JN.1*
69	24032		Taranto	Taranto Gennarini	6,28E+03	Package C	Omicron JN.1*
70	24068		Trapani	Trapani	1,44E+04	
71	24069		Trapani	Mazara del Vallo	3,42E+03	
72	24070		Trapani	Marsala	1,14E+04	
73	24127		Ragusa	Modica	5,53E+03	Package C	Omicron JN.1*
74	24128		Ragusa	Ragusa	2,93E+03	Package C	Omicron JN.1*
75	24130		Ragusa	Vittoria	6,20E+03	Package C	Omicron JN.1*
76	24131	Sicilia	Caltanissetta	Gela Macchitella	6,93E+03	Package C	Omicron JN.1*
77	24150		Palermo	Acqua dei Corsari	2,16E+04	
78	24151		Palermo	Fondo Verde	4,35E+04	
79	24152		Caltanissetta	Caltanissetta e San Cataldo	8,01E+04	Package B + F157L + L452R	Omicron XBB.1.5*/XBB.1.9*
100	24182		Catania	Pantano d'Arci	1,28E+04	
101	24183		Catania	Giarre	8,63E+03	
102	24184		Siracusa	Siracusa	1,40E+04	Package C	Omicron JN.1*
108	24244	Toscana	Lucca	Pontetetto	1,35E+04	

109	24243		Pisa	Pisa Nord - S. Jacopo	<LOD	Package C	Omicron JN.1*
80	24116	Umbria	Perugia	Perugia - Pian della Genna	1,35E+04		
81	24025		Padova	Padova Ca' Nordio - centro storico	4,81E+04		
82	24026		Padova	Padova Ca' Nordio - zip	1,57E+04	Package C	Omicron JN.1*
83	24027		Padova	Padova Guizza	4,26E+03		
84	24028		Padova	Abano Terme	1,66E+04	Package C	Omicron JN.1*
85	24036	Veneto	Treviso	Treviso	8,09E+03	Package C	Omicron JN.1*
86	24037		Venezia	Venezia Fusina	5,99E+03		
87	24038		Vicenza	Vicenza Casale	1,55E+03		
88	24104		Verona	Verona_collettore 1M	1,36E+03	Package A + L455F	Omicron XBB.1.5*/XBB.1.9*
89	24105		Verona	Verona_collettore 3M	5,58E+02	Package C	Omicron JN.1*
90	24106		Verona	Verona_collettore 8M	1,07E+03	Package C	Omicron JN.1*

NA= not available

[a] 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: 26/01/2024).

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

[c] 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	DEL69/70	V83A	V127F	G142D	DEL 144	H146Q/H146K	F157S	R158G	Q183E	DEL211/212	V213G	V213E	L216F	H245N	G252V	A264D	I332V	G339H	R346T	K356T	L368I	S371F	S373P	S375F	T376A	R403K	D405N	R408S	K417N	N440K	V445H	V445P	G446S	N450D	L452W	L455S	F456L	N460K	VARIANTS
88			■	■	■	■	■			■			■			■			■	■		■	■	■	■	■		■	■	■	■		■	■					■	Package A (Omicron XBB.1.5*/XBB.1.9*)
17, 23, 43, 45, 52, 79	■		■	■	■	■	■			■			■			■			■	■		■	■	■	■	■		■	■	■	■		■	■				■	■	Package B (Omicron XBB.1.5*/XBB.1.9*)
1, 15, 20, 27, 28, 29, 39, 48, 50, 51, 56, 57, 65, 68, 69, 73, 74, 75, 76, 82, 84, 85, 89, 90, 102, 108		■	■	■	■	■		■	■		■	■		■	■		■	■	■		■		■	■	■	■	■	■	■	■	■	■			■	■	■		■	Package C (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 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 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 JN.1* lineage is prevalent in Italy in January 2024. Additionally, the Omicron XBB.1.5*/XBB.1.9* lineages have also been 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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