Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
33rd Report
(Study period: May 6th to May 10th, 2024)

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Main findings:

- During the week of 6 May to 10 May 2024, a total of 99 wastewater samples were collected from 16 Regions and 2 Autonomous Province (A.P.).
- Mutations characteristic of the Omicron variant were identified in 3 Regions/A.P., while no sequencing data were collected from the remaining areas.
- Analysis of the sequences obtained by Sanger sequencing showed that 83.3% of the positive samples had amino acid substitutions typical of the Omicron JN.1* lineage including those with R346T and F456L mutations, possibly associated with the KP2* variant. The remaining 16.7% of the samples had amino acid substitutions of 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 33rd national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from 6 to 10 May 2024.

Methodology

The 33rd national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out in Italy from 6 to 10 May 2024. The survey involved the collection of 99 wastewater samples from 95 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]. 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 amplification of 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.

Results

Real Time qPCR

Of the 83 samples analysed, a total of 61 (73.5%) 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 1.00E + 02 to 4.54E + 04 genome copies (g.c.) per liter of sewage.

Sanger Sequencing

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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

Table 1 summarises the results of the long-nested PCR assay and sequencing methods. A total of 6 samples (6.1%) from 4 Regions/AP were successfully amplified using the long-nested PCR assay described above. High-quality sequences were obtained from 6 of these samples by Sanger sequencing. Sanger sequencing confirmed that all the sequences corresponded to the Omicron variant.

Analysis of the wastewater samples revealed the presence of two SARS-CoV-2 lineages, as shown in Tables 1 and 2. The Omicron JN.1* lineage was detected in 83.3% of the positive samples (5 samples), followed by the Omicron XBB.1.5/1.9* lineages (which are not distinguishable in the sequenced region), detected in 16.7% (1 samples). Within the JN.1* lineage, 75% of the samples (3 samples) can be distinguished as KP.2* sublineage by the presence of the R346T and F456L mutations.

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
- **Package B (assigned to the lineage Omicron XBB.1.5*/XBB.1.9*)**= V83A, G142D, DEL144, H146Q, Q183E, V213E, G252V, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, F456L, N460K

The Omicron JN.1* lineage was detected in Veneto and in the A.Ps of Bolzano and Trento. The Omicron JN.1* variant in association with R346T and F456L mutations , probably indicating the presence of the sublineage KP.2* sublineage, was found both in both A.P. of Trento and Bolzano. Key mutations of the Omicron XBB.1.5*/XBB.1.9* were found in Emilia-Romagna.

Table 1. PCR and sequencing results

ID ISS	ID SARI	Region	City	WTP	RT-qPCR (c.g./L)	Mutations found by Sanger sequencing (long PCR ID_1034)	SARS-CoV-2 lineages (Sanger sequencing)
1	25687	Abruzzo	L'Aquila	Pile	<LOD		
2	25688		Teramo	Villa Pavone	<LOD		
3	25689		Pescara	Via Raiale	<LOD		
4	25690		Pescara	Villa Carmine	<LOD		
5	25691		Chieti	S. Martino	<LOD		
87	25827	Basilicata	Potenza	Tiera di Vaglio	2,04E+03		
88	25828		Matera	Pantano	1,35E+03		
81	25831	Campania	Napoli	Napoli OVEST - Ingresso Principale	3,45E+03		
82	25832		Napoli	Napoli OVEST - ex ingresso Camaldoli	1,21E+04		
83	25833		Napoli	Napoli EST	<LOD		
6	25610	Emilia-Romagna	Piacenza	Borgoforte	<LOD		
7	25611		Parma	Parma ovest	<LOD		
8	25612		Reggio Emilia	Mancasale	<LOD		
9	25646		Ferrara	Ferrara - Linea 1	<LOD		
10	25647		Ferrara	Ferrara - Linea 2	<LOD		
89	25770		Bologna	IDAR	7,25E+03		
90	25771		Forlì-Cesena	Forlì	5,50E+03		
91	25772		Forlì-Cesena	Cesena	1,08E+04		
92	25773		Ravenna	Faenza	5,50E+03	Package B	Omicron XBB.1.5*/XBB.1.9*
93	25774		Bologna	Imola	6,25E+03	Package A + R346T + R355M + F456L [a]	Omicron JN.1* + R346T + R355M + F456L
94	25776		Rimini - Forlì-Cesena	S. Giustina	1,15E+04		
97	26041	Friuli Venezia Giulia	Udine	Udine	3,84E+03		
98	26042		Pordenone	Cordenons	6,25E+03		
99	26043		Trieste	Servola	3,91E+03		
11	25592	Lazio	Roma	Civitavecchia Fiumaretta	<LOD		

12	25613	Liguria	Genova	Recco	<LOD
13	25625		Genova	Pegli	1,10E+04
14	25626		Genova	Voltri	<LOD
15	25627		Genova	Quinto	3,20E+03
16	25628		Genova	Rapallo	1,31E+04
17	25629		Genova	Sestri P	1,11E+04
18	25630		Genova	Sturla	7,17E+03
19	25631		Genova	Darsena	3,76E+03
20	25632		Genova	Punta Vagno Genova	9,90E+03
21	25633		Genova	Valpolcevera	4,03E+03
22	25634		La Spezia	La Spezia	4,54E+04
23	25635		Imperia	Sanremo - località Capo Verde	1,23E+04
24	25636		Savona	Savona	3,75E+03
25	25637		Imperia	Imperia	1,09E+04
26	25638		Savona	Borghetto Santo Spirito	3,77E+03
27	25620	Lombardia	Bergamo	Bergamo	<LOD
28	25621		Brescia	Verziano	2,20E+04
29	25623		Cremona	Citta di Cremona	1,02E+04
70	25593		Milano	Milano Nosedo	NA
71	25594		Milano	Milano San Rocco	NA
72	25595		Como	Como	NA
73	25596		Pavia	Pavia	NA
74	25597		Como - Lecco - Milano - Monza e della Brianza	Monza	NA
75	25598		Pavia	Vigevano	NA
76	26016		Sondrio	Sondrio	<LOD
30	25668	Marche	Pesaro-Urbino	Borgheria	1,64E+03
31	25669		Pesaro-Urbino	Ponte Metauro	1,11E+03
32	25670		Ancona	Zipa	5,95E+03
33	25671		Ancona	Falconara	1,17E+03
34	25692	Molise	Campobasso	Termoli - località Porto	<LOD
35	25693		Campobasso	Termoli - località Pantano Basso	1,56E+02

36	25694		Campobasso	Campobasso - San Pietro	<LOD		
78	25727	P.A. Bolzano	Bolzano	IDA Bolzano	4,82E+03		
79	25728		Bolzano	IDA Merano	1,05E+03		
80	25729		Bolzano	IDA Termeno	4,91E+03	Package A + R346T + R355M + F456L [a]	Omicron JN.1* + R346T + F456L
37	25681	P.A. Trento	Trento	Trento nord	7,79E+03		
38	25682		Trento	Trento sud	3,80E+04	Package A + R346T + F456L [a]	Omicron JN.1* + R346T + F456L
39	25683		Trento	Rovereto	2,47E+04	Package A + R346T + F456L [a]	Omicron JN.1* + R346T + F456L
40	25540	Piemonte	Torino	Castiglione Torinese	7,83E+02		
41	25541		Biella	Biella Nord	2,25E+02		
42	25542		Biella	Biella Sud	<LOD		
43	25543		Novara	Novara	2,00E+03		
44	25589		Alessandria	Alessandria	<LOD		
45	25590		Asti	Asti	1,96E+03		
46	25591		Cuneo	Cuneo	2,17E+03		
47	25585	Puglia	Bari	Bari Est	NA		
48	25586		Bari	Bari Ovest	NA		
49	25587		Taranto	Taranto Bellavista	NA		
50	25588		Taranto	Taranto Gennarini	NA		
51	25578	Sicilia	Trapani	Trapani	1,20E+03		
52	25579		Trapani	Mazara del Vallo	9,28E+02		
53	25654		Ragusa	Ragusa	1,00E+02		
54	25655		Ragusa	Modica	<LOD		
55	25656		Caltanissetta	Gela Macchitella	<LOD		
56	25664		Palermo	Acqua dei Corsari	2,18E+03		
57	25665		Palermo	Fondo Verde	1,27E+03		
58	25666		Caltanissetta	Caltanissetta e San Cataldo	2,01E+03		
59	25667		Palermo	Bagheria	1,59E+03		
84	25599		Catania	Pantano d'Arci	4,77E+03		
85	25600		Catania	Giarre	5,04E+03		
86	25601		Siracusa	Siracusa	4,13E+03		

95	26028	Toscana	Pisa	Pisa Nord - S. Jacopo	NA		
96	26029		Lucca	Pontetetto	NA		
77	25605	Umbria	Perugia	Perugia - Pian della Genna	1,79E+04		
60	25574	Veneto	Padova	Padova Ca' Nordio - centro storico	NA		
61	25575		Padova	Padova Ca' Nordio - zip	NA		
62	25576		Padova	Padova Guizza	NA		
63	25577		Padova	Abano Terme	NA		
64	25580		Treviso	Treviso	5,55E+03		
65	25581		Vicenza	Vicenza Casale	5,12E+03		
66	25582		Venezia	Venezia Fusina	2,39E+04		
67	25615		Verona	Verona_collettore 1M	2,46E+04		
68	25616		Verona	Verona_collettore 3M	1,64E+04	Package A (Partial sequence, from G142D to G339H) [b]	Omicron JN.1*
69	25617		Verona	Verona_collettore 8M	4,65E+03		

[a] The key mutations of Omicron JN.1* in association with R346T and F456L may indicate the presence of the Omicron KP.2* sublineage.

[b] 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	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
38, 39, 80, 93	■		■	■	■		■	■		■	■		■	■		■	■	■	■	■		■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	**Package A (Omicron JN.1* + R346T + F456L)**
68	■		■	■	■		■	■		■	■		■	■		■	■	■		■		■	■	■	■	■	■	■	■	■	■		■	■	■	■		■	**Package A (Omicron JN.1*)**
92		■		■	■	■			■			■			■			■	■		■	■	■	■	■		■	■	■	■		■	■				■	■	**Package B (Omicron XBB.1.5*/XBB.1.9*)**

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* lineages being the most prevalent. 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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