Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
35th Report
(Study period: July 1st to July 5th, 2024)

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Main findings:

- During the week of 1 July to 5 July 2024, a total of 102 wastewater samples were collected from 16 Regions and 2 Autonomous Province (A.P.).
- Mutations characteristic of the Omicron variant were identified in 5 regions/A.P., while sequencing data were not availabe from the remaining regions.
- Sanger sequencing analysis showed that 100% of the positive samples carried amino acid substitutions typical of the Omicron JN.1* lineage, including mutations associated with the KP.2*, KP.3*, LB.1* and JN.1.18* 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 35th national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from 1 to 5 July 2024.

Methodology

The 35th national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out in Italy from 1 to 5 July 2024. The survey involved the collection of 102 wastewater samples from 98 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 only 68 of the 102 samples. Out of the 68 samples analysed, a total of 60 (88%) 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 7.99E + 01 to 1.46E + 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 7/102 samples (6.9%) from 5 Regions/AP 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 five regions/A.P.: Emilia-Romagna, Liguria, Sicilia, Veneto, and the Autonomous Province of Bolzano. Within this lineage, 42.9% of the samples (3/7 samples) showed mutation associated with the KP.3*/JN.1.16* sublineage, 28.6% (2/7 samples)

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

with the KP.2* sublineage, 14.3% (1/7 sample) with the JN.1.18* sublineage and 14.3% (1/7 sample) to the LB.1* sublineage.
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	City	WTP	RT-qPCR (c.g./L)	Mutations found by Sanger sequencing (long PCR ID_1034)	SARS-CoV-2 lineages (Sanger sequencing)
95	26403	Abruzzo	Teramo	Villa Pavone	9,39E+01		
96	26404		L'Aquila	Pile	4,22E+02		
97	26405		Pescara	Villa Carmine	2,03E+02		
98	26406		Pescara	Via Raiale	<LOD		
99	26407		Chieti	S. Martino	<LOD		
100	26455	Campania	Napoli	Napoli OVEST - Ingresso Principale	2,25E+04		
101	26456		Napoli	Napoli OVEST - ex ingresso Camaldoli	2,11E+04		
102	26454		Napoli	Napoli EST	1,17E+04		
1	26239	Emilia-Romagna	Piacenza	Borgoforte	1,21E+02		
2	26240		Parma	Parma ovest	<LOD		
3	26241		Reggio Emilia	Mancasale	3,80E+02		
4	26342		Ferrara	Ferrara - Linea 1	<LOD		
5	26343		Ferrara	Ferrara - Linea 2	3,80E+04		
6	26344		Modena	Carpi	2,88E+04		
89	26480		Modena	Naviglio	1,18E+03		
90	26529		Bologna	Imola	1,90E+03		
91	26562		Forlì-Cesena	Forlì	6,00E+02		
92	26563		Forlì-Cesena	Cesena	3,25E+02	Package A + Q183H + R346T + F456L[a]	Omicron JN.1*
93	26564		Bologna	IDAR	1,10E+02		
94	26565		Ravenna - Forlì-Cesena	Ravenna	3,25E+02		
103	26566		Rimini - Forlì-Cesena	S. Giustina	1,65E+03		
104	26417	Friuli-Venezia Giulia	Pordenone	Cordenons	<LOD		
105	26418		Trieste	Servola	1,02E+04		
7	26295	Lazio	Roma	Civitavecchia Fiumaretta	<LOD		
72	26349		Viterbo	Strada Bagni	NA		
73	26350		Aprilia	Aprilia-Via del campo	NA		
74	26351		Latina	Latina Est	NA		
75	26352		Anzio	Anzio-Colle Cocchino	NA		

76	26353		Pomezia	Pomezia Capoluogo	NA		
77	26354		Velletri	La Chiusa-Velletri	NA		
78	26355		Tivoli	Ponte Lucano di Guidonia	NA		
8	26242	Liguria	Genova	Recco	1,18E+03	Package A + F456L[b]	Omicron JN.1*
9	26257		Genova	Pegli	NA		
10	26258		Genova	Voltri	NA		
11	26259		Genova	Quinto	NA	Package A + R346T + F456L[c]	Omicron JN.1*
12	26260		Genova	Sestri P	NA		
13	26261		Genova	Sturla	NA		
14	26262		Genova	Darsena	NA		
15	26263		Genova	Punta Vagno Genova	NA		
16	26264		Genova	Valpolcevera	NA		
17	26265		La Spezia	La Spezia	NA		
18	26266		Savona	Savona	NA		
19	26267		Imperia	Imperia	NA		
20	26268		Imperia	Sanremo - località Capo Verde	NA		
21	26269		Savona	Borghetto Santo Spirito	NA		
22	26271		Genova	Rapallo	NA		
23	26273	Lombardia	Bergamo	Bergamo	1,76E+04		
24	26274		Brescia	Verziano	<LOD		
25	26276		Cremona	Città di Cremona	1,77E+04		
79	26251		Milano	Milano Nosedo	NA		
80	26252		Milano	Milano San Rocco	NA		
81	26253		Como	Como	NA		
82	26255		Como - Lecco - Milano - Monza e della Brianza	Monza	NA		
83	NA		Sondrio	Sondrio	NA		
26	26296	Marche	Pesaro-Urbino	Borgheria	1,18E+04		
27	26297		Pesaro-Urbino	Ponte Metauro	3,99E+03		
28	26298		Ancona	Zipa	6,46E+03		
29	26299		Ancona	Falconara	1,30E+03		
84	26374	P.A. Bolzano	Bolzano	IDA Merano	1,46E+05		
85	26375		Bolzano	IDA Termeno	2,13E+04	Package A + R346T + F456L[c]	Omicron JN.1*
86	26373		Bolzano	IDA Bolzano	6,59E+04		
30	26248	P.A. Trento	Trento	Trento nord	1,03E+04		
31	26249		Trento	Trento sud	1,25E+04		
32	26250		Trento	Rovereto	1,68E+04		

33	26209	Piemonte	Torino	Castiglione Torinese	7,63E+02		
34	26210		Biella	Biella Nord	8,20E+02		
35	26211		Biella	Biella Sud	7,50E+02		
36	26212		Novara	Novara	3,50E+02		
37	26244		Alessandria	Alessandria	3,13E+04		
38	26245		Asti	Asti	1,35E+04		
39	26246		Cuneo	Cuneo	2,53E+04		
40	26224	Puglia	Bari	Bari Est	<LOD		
41	26225		Bari	Bari Ovest	9,21E+01		
42	26226		Taranto	Taranto Bellavista	3,00E+02		
45	26229		Taranto	Taranto Gennarini	1,75E+02		
46	26233	Sicilia	Trapani	Trapani	9,85E+03		
47	26234		Trapani	Mazara del Vallo	4,88E+03		
48	26235		Trapani	Marsala	1,30E+04	Package A + F59S + R346T[d]	Omicron JN.1*
49	26290		Catania	Pantano d'Arci	3,71E+03		
50	26291		Catania	Giarre	6,19E+03		
51	26292		Siracusa	Siracusa	1,45E+03		
52	26321		Caltanissetta	Gela Macchitella	3,03E+03		
53	26322		Ragusa	Modica	7,00E+02		
54	26323		Ragusa	Ragusa	2,25E+02		
55	26324		Ragusa	Vittoria	9,50E+02		
56	26335		Palermo	Acqua dei Corsari	2,83E+04		
57	26336		Palermo	Fondo Verde	7,59E+03		
58	26337		Palermo	Bagheria	6,03E+03		
59	26338		Caltanissetta	Caltanissetta e San Cataldo	1,67E+04		
87	26359	Toscana	Lucca	Pontetetto	7,99E+01		
88	26360		Pisa	Pisa Nord - S. Jacopo	1,41E+03		
60	26218	Umbria	Perugia	Perugia - Pian della Genna	1,90E+04		
61	26213	Veneto	Padova	Padova Ca' Nordio - centro storico	NA		
62	26214		Padova	Padova Ca' Nordio - zip	NA		
63	26215		Padova	Padova Guizza	NA		
64	26216		Padova	Abano Terme	NA		
65	26236		Treviso	Treviso	3,69E+04		
66	26237		Venezia	Venezia Fusina	5,55E+04	Package A + F456L[b]	Omicron JN.1*
67	26238		Vicenza	Vicenza Casale	6,10E+03		
68	26279		Verona	Verona_collettore 1M	1,06E+05	Package A + F456L[b]	Omicron JN.1*

69	26280		Verona	Verona_collettore 3M	9,53E+04
70	26281		Verona	Verona_collettore 8M	4,33E+04

NA= Not available

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

[b] 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.

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

[d] The key mutations of Omicron JN.1* in association with F59S and R346T may indicate the presence of the Omicron JN.1.18* sublineage.

Table 2. Sanger sequencing results

ID SAMPLES	F59S	DEL69/70	V127F	G142D	DEL 144	F157S	R158G	Q183H	DEL211/212	V213G	L216F	H245N	A264D	I332V	G339H	R346T	K356T	S371F	S373P	S375F	T376A	R403K	D405N	R408S	K417N	N440K	V445H	G446S	N450D	L452W	L455S	F456L	N460K	VARIANTS
92		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	**Package A (Omicron JN.1* + Q183H + R346T + F456L)**
8, 66, 68		■	■	■	■	■	■		■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	**Package A (Omicron JN.1* + F456L)**
11, 85		■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	**Package A (Omicron JN.1* + R346T + F456L)**
48	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	**Package A (Omicron JN.1* +F59S + R346T)**

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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