Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
32nd Report
(Study period: April 8th to April 12th, 2024)

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Main findings:

- During the week of 8 April to 12 April 2024, a total of 100 wastewater samples were collected from 16 Regions and 2 Autonomous Province (A.P.).
- Mutations characteristic of the Omicron variant were identified in 2 Regions/A.P., 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.
- Next-generation sequencing (NGS) results confirmed the widespread presence of the key mutation of the JN.1* lineage, but also the presence in one sample of the JN.1* lineage with two additional mutations, R346T and F456L, which may indicate the presence of Omicron KP.2*.

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 32nd national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from 8 to 12 April 2024.

Methodology

The 32nd national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out in Italy from 8 to 12 April 2024. The survey involved the collection of 100 wastewater samples from 96 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. However, due to the rapid spread of the new KP.2* variant (also known as FLiRT), NGS sequencing was also performed on individual samples. This was done to check for the possible minority presence of this lineage. NGS was performed using the Oxford Nanopore platform. Bioinformatic analysis was

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carried out on the sequencing data obtained, and variant calling was performed for FLiRT variantfollowing established protocol as previously described[3].
For variant classification, we adopted a lineage classification based on 'outbreak.info'[4] 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 93 samples analysed, a total of 51 (54.8%) 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 5.75E + 01 to 6.89E + 04 genome copies (g.c.) per liter of sewage.

Sanger and NGS Sequencing

Table 1 summarises the results of the long-nested PCR assay and sequencing methods. A total of 2 samples (2%) from 2 Region/A.P. were successfully amplified using the long-nested PCR assay described above. Sanger sequencing confirmed that all the sequences corresponded to the Omicron variant.
Analysis of the wastewater samples revealed the presence of one SARS-CoV-2 lineage, as shown in Tables 1 and 2. Specifically, the Omicron JN.1* lineage was detected in 100% of the positive samples (2 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* lineage was found in Emilia-Romagna and A.P. Bolzano. In addition, the key mutations of Omicron JN.1* in association with R346T and F456L (probably indicating the presence of the KP.2* sublineage) have also been found by NGS sequencing in the Emilia-Romagna region.

[3] La Rosa, G.; Brandtner, D.; Mancini, P.; Veneri, C.; Bonanno Ferraro, G.; Bonadonna, L.; Lucentini, L.; Suffredini, E. Key SARS-CoV-2 Mutations of Alpha, Gamma, and Eta Variants Detected in Urban Wastewaters in Italy by Long-Read Amplicon Sequencing Based on Nanopore Technology. Water 2021, 13, 2503. https://doi.org/10.3390/w13182503

[4] https://outbreak.info/situation-reports, date: 05/12/2023

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)	Mutations found by NGS	SARS-CoV-2 variant (NGS)
79	25383	Abruzzo	Pescara	Via Raiale	<LOD				
80	25382	Abruzzo	Pescara	Villa Carmine	<LOD				
81	25392	Abruzzo	Chieti	S. Martino	<LOD				
82	25394	Abruzzo	L'Aquila	Pile	<LOD				
83	25393	Abruzzo	Teramo	Villa Pavone	<LOD				
65	25373	Basilicata	Matera	Pantano	2,79E+04				
66	25372	Basilicata	Potenza	Tiera di Vaglio	<LOD				
99	25432	Campania	Napoli	Napoli OVEST - Ingresso Principale	2,61E+03				
100	25431	Campania	Napoli	Napoli EST	<LOD				
101	25433	Campania	Napoli	Napoli OVEST - ex ingresso Camaldoli	<LOD				
1	25252	Emilia-Romagna	Piacenza	Borgoforte	5,45E+03				
2	25253	Emilia-Romagna	Parma	Parma ovest	<LOD				
3	25254	Emilia-Romagna	Reggio Emilia	Mancasale	<LOD				
4	25306	Emilia-Romagna	Ferrara	Ferrara - Linea 1	<LOD				
5	25307	Emilia-Romagna	Ferrara	Ferrara - Linea 2	<LOD				
68	25468	Emilia-Romagna	Bologna	IDAR	1,25E+03	Package A + R346T	Omicron JN.1*	• Package A • Package A + R346T + F456L[a]	• Omicron JN.1* • Omicron JN.1*+ R346T + F456L
67	25467	Emilia-Romagna	Forlì-Cesena	Cesena	5,00E+02				
69	25469	Emilia-Romagna	Modena	Naviglio	5,00E+02				
70	25470	Emilia-Romagna	Ravenna	Faenza	1,25E+02				
71	25471	Emilia-Romagna	Bologna	Imola	1,28E+03				

72	25472	Emilia-Romagna	Ravenna - Forlì-Cesena	Ravenna	2,08E+03
73	25473	Emilia-Romagna	Forlì-Cesena	Forlì	4,75E+02
74	25474	Emilia-Romagna	Rimini - Forlì-Cesena	S. Giustina	8,50E+02
102	25237	Friuli-Venezia Giulia	Pordenone	Cordenons	<LOD
103	25238	Friuli-Venezia Giulia	Udine	Udine	2,20E+03
104	25239	Friuli-Venezia Giulia	Trieste	Servola	<LOD
97	25434	Lazio	Roma	Civitavecchia Fiumaretta	<LOD
6	25255	Liguria	Genova	Recco	5,13E+03
7	25282	Liguria	Genova	Pegli	5,40E+03
9	25284	Liguria	Genova	Quinto	<LOD
8	25283	Liguria	Genova	Voltri	3,32E+03
11	25286	Liguria	Genova	Sestri P	<LOD
12	25287	Liguria	Genova	Sturla	1,33E+04
13	25288	Liguria	Genova	Darsena	8,98E+03
14	25289	Liguria	Genova	Punta Vagno Genova	2,31E+03
15	25290	Liguria	Genova	Valpolcevera	2,53E+03
16	25291	Liguria	La Spezia	La Spezia	3,16E+03
10	25285	Liguria	Genova	Rapallo	7,70E+03
18	25293	Liguria	Savona	Borghetto Santo Spirito	<LOD
19	25294	Liguria	Savona	Savona	<LOD
17	25292	Liguria	Imperia	Sanremo - località Capo Verde	5,33E+03
20	25301	Lombardia	Bergamo	Bergamo	<LOD
21	25302	Lombardia	Cremona	Citta di Cremona	<LOD
22	25304	Lombardia	Brescia	Verziano	<LOD
61	25248	Lombardia	Como	Como	NA
98	NA	Lombardia	Sondrio	Sondrio	NA

59	25246	Lombardia	Milano	Milano Nosedo	NA					
60	25247	Lombardia	Milano	Milano San Rocco	NA					
62	25249	Lombardia	Pavia	Pavia	NA					
63	25250	Lombardia	Como - Lecco - Milano - Monza e della Brianza	Monza	NA					
64	25251	Lombardia	Pavia	Vigevano	NA					
24	25348	Marche	Pesaro-Urbino	Ponte Metauro	<LOD					
26	25350	Marche	Ancona	Falconara	7,95E+03					
23	25347	Marche	Pesaro-Urbino	Borgheria	3,37E+03					
25	25349	Marche	Ancona	Zipa	1,46E+04					
84	25396	Molise	Campobasso	Termoli - località Pantano Basso	<LOD					
85	25395	Molise	Campobasso	Termoli - località Porto	<LOD					
86	25397	Molise	Campobasso	Campobasso - San Pietro	<LOD					
75	25354	P.A. Bolzano	Bolzano	IDA Bolzano	5,56E+03	Package A	Omicron JN.1*	•	Package A	• Omicron JN.1*
76	25355	P.A. Bolzano	Bolzano	IDA Merano	2,13E+03					
77	25353	P.A. Bolzano	Bolzano	IDA Termeno	1,13E+03					
27	25243	P.A. Trento	Trento	Trento nord	2,98E+03					
28	25244	P.A. Trento	Trento	Trento sud	3,47E+03					
29	25245	P.A. Trento	Trento	Rovereto	3,82E+03					
30	25193	Piemonte	Torino	Castiglione Torinese	<LOD					
31	25194	Piemonte	Biella	Biella Nord	<LOD					
32	25195	Piemonte	Biella	Biella Sud	<LOD					
33	25196	Piemonte	Novara	Novara	<LOD					
34	25222	Piemonte	Alessandria	Alessandria	4,43E+03					
36	25224	Piemonte	Cuneo	Cuneo	<LOD					
35	25223	Piemonte	Asti	Asti	1,36E+03					
37	25212	Puglia	Bari	Bari Est	<LOD					
38	25215	Puglia	Bari	Bari Ovest	<LOD					

39	25218	Puglia	Taranto	Taranto Bellavista	4,66E+02
40	25221	Puglia	Taranto	Taranto Gennarini	<LOD
41	25199	Sicilia	Catania	Pantano d'Arci	<LOD
42	25200	Sicilia	Catania	Giarre	<LOD
43	25201	Sicilia	Siracusa	Siracusa	9,65E+02
44	25202	Sicilia	Trapani	Trapani	1,13E+03
46	25310	Sicilia	Ragusa	Ragusa	7,50E+01
47	25311	Sicilia	Ragusa	Vittoria	<LOD
48	25312	Sicilia	Caltanissetta	Gela Macchitella	5,75E+01
45	25203	Sicilia	Trapani	Mazara del Vallo	2,25E+03
89	25378	Sicilia	Palermo	Acqua dei Corsari	1,45E+03
90	25379	Sicilia	Palermo	Fondo Verde	1,14E+03
91	25380	Sicilia	Caltanissetta	Caltanissetta e San Cataldo	1,88E+03
92	25381	Sicilia	Palermo	Bagheria	1,22E+03
87	25363	Toscana	Pisa	Pisa Nord - S. Jacopo	<LOD
88	25365	Toscana	Lucca	Pontetetto	<LOD
78	25388	Umbria	Perugia	Perugia - Pian della Genna	<LOD
49	25145	Veneto	Padova	Padova Ca' Nordio - centro storico	6,89E+04
50	25146	Veneto	Padova	Padova Ca' Nordio - zip	<LOD
51	25147	Veneto	Padova	Padova Guizza	<LOD
52	25148	Veneto	Padova	Abano Terme	2,16E+04
53	25204	Veneto	Treviso	Treviso	5,77E+03
54	25205	Veneto	Vicenza	Vicenza Casale	3,35E+02
55	25206	Veneto	Venezia	Venezia Fusina	5,72E+03
57	25279	Veneto	Verona	Verona_collettore 3M	4,42E+03
58	25281	Veneto	Verona	Verona_collettore 8M	1,04E+03
56	25278	Veneto	Verona	Verona_collettore 1M	8,30E+02

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

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
68, 75																														**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. It is important to note that only two samples were successfully sequenced in April 2024, despite repeated PCR attempts, suggesting that the low viral concentrations detected during that period may have hindered the amplification of a longer fragment by nested PCR.

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. However, using NGS sequencing, it was also possible to detect the minority presence of the KP.2* lineage in a single sample. 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. In this flash survey, in addition to sanger sequencing, next-generation sequencing (NGS) was introduced to detect the presence of FLiRT variant mutations in minority populations, which might have been missed by Sanger sequencing. This approach allowed, for the first time in Italy, the identification of the FLiRT variant in wastewater.

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