Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
37th Report
(Study period: September 2nd to September 6th, 2024)

Edited by:

- Giuseppina La Rosa, Giusy Bonanno Ferraro, Pamela Mancini, Carolina Veneri, Agata Franco, Marcello Iaconelli, Luca Lucentini (National Center for Water Safety, Istituto Superiore di Sanità)
- David Brandtner (Department of Infectious Diseases, Istituto Superiore di Sanità)
- Giuseppe D'Avenio, Mirko Rossi (National Center for Innovative Technologies in Public Health, Istituto Superiore di Sanità)
- Elisabetta Suffredini (Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità

Main findings:

- During the week of 2 September to 6 September 2024, a total of 103 wastewater samples were collected from 16 Regions and 2 Autonomous Province (A.P.).
- Mutations characteristic of the Omicron variant were identified in 7 regions/A.P., while sequencing data were not available 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.3*, KP.2* and LB.1* 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 37th national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, conducted from 2 to 6 September 2024.

Methodology

The 37th national Flash Survey on SARS-CoV-2 variants in wastewater samples was carried out in Italy from 2 to 6 September 2024. The survey involved the collection of 103 wastewater samples from 99 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 88 of the 103 samples. Out of the 88 samples analysed, a total of 78 (89%) 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 2.96E + 01 to 7.97E + 04 genome copies (g.c.) per liter of sewage.

Sanger Sequencing

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

Table 1 summarises the results of the long-nested PCR assay and sequencing. A total of 13/103 samples (12.6%) from 7 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 7 regions/A.P.: Emilia-Romagna, Liguria, Piemonte, Sicilia, Veneto and the A.Ps of Bolzano and Trento.

Within the JN.1* lineage, 69.2% of the samples (9/13 samples) showed mutation associated with the KP.3*/JN.1.16* sublineage, 23.1% (3/13 samples) with the KP.2* sublineage and 7.7% (1/13 sample) with 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/A.P.	City	WTP	RT-qPCR (c.g./L)	Mutations found by Sanger sequencing (long PCR ID_1034)	SARS-CoV-2 lineages (Sanger sequencing)
71	27093	Abruzzo	Teramo	Villa Pavone	2,49E+02		
72	27094		L'Aquila	Pile	9,58E+02		
73	27097		Pescara	Villa Carmine	3,94E+02		
74	27096		Pescara	Via Raiale	3,44E+02		
75	27095		Chieti	S. Martino	<LOD		
99	27336	Campania	Napoli	Napoli OVEST - Ingresso Principale	1,01E+04		
100	27335		Napoli	Napoli EST	3,38E+03		
101	27337		Napoli	Napoli OVEST - ex ingresso Camaldoli	<LOD		
2	26984	Emilia-Romagna	Ferrara	Ferrara - Linea 1	1,10E+04	Package A + F456L[a]	Omicron JN.1*
3	26985		Ferrara	Ferrara - Linea 2	<LOD		
4	26986		Modena	Carpi	8,00E+02		
58	26883		Piacenza	Borgoforte	<LOD		
59	26884		Parma	Parma ovest	4,38E+03		
60	26885		Reggio Emilia	Mancasale	2,39E+03		
91	27158		Forlì-Cesena	Forlì	2,03E+03		
92	27163		Forlì-Cesena	Cesena	2,80E+03		
93	27159		Modena	Naviglio	1,40E+03		
94	27160		Bologna	IDAR	5,33E+03	Package A + F456L[a]	Omicron JN.1*
95	27161		Ravenna - Forlì-Cesena	Ravenna	4,33E+03	Package A + F456L[a]	Omicron JN.1*
96	27162		Bologna	Imola	2,75E+03	Package A + F59L+ F456L[a]	Omicron JN.1*
97	27266		Ravenna	Faenza	5,25E+02		
98	27268		Rimini - Forlì-Cesena	S. Giustina	4,55E+03	Package A + F59L + H146del + R346T + F456L[b]	Omicron JN.1*
52	27181	Friuli-Venezia Giulia	Udine	Udine	1,14E+04		
53	27182		Trieste	Servola	4,96E+03		

54	27183		Pordenone	Cordenons	6,10E+03		
7	27004		Roma	Civitavecchia Fiumaretta	<LOD		
62	27440		Viterbo	Viterbo - Strada Bagni	NA		
63	27441		Roma	Anzio - Colle Cocchino	NA		
64	27442	Lazio	Latina	Aprilia (Via del Campo)	NA		
65	27443		Latina	Latina Loc Latina Est	NA		
66	27444		Roma	Pomezia - Via Cincinnato	NA		
67	27445		Roma	Velletri (LA CHIUSA-SORBO)	NA		
9	27005		Genova	Darsena	1,55E+04		
10	27006		Genova	Sturla	2,62E+04	Package A + S256L + F456L[a]	Omicron JN.1*
11	27007		La Spezia	La Spezia	7,97E+04		
12	27008		Savona	Savona	9,39E+03		
13	27009		Genova	Valpolcevera	1,20E+04	Package A + R346T + F456L[b]	Omicron JN.1*
14	27010		Genova	Pegli	1,69E+04		
16	27012	Liguria	Genova	Quinto	1,53E+04		
17	27013		Genova	Sestri P	6,02E+03		
18	27014		Savona	Borghetto Santo Spirito	1,74E+04		
19	27015		Genova	Voltri	1,84E+04		
20	27016		Genova	Sestri P	6,02E+03		
21	27017		Genova	Punta Vagno Genova	9,13E+03		
61	26886		Genova	Recco	5,03E+03		
46	NA		Bergamo	Bergamo	NA		
47	NA		Brescia	Verziano	NA		
102	NA		Mantova	Mantova	NA		
103	NA		Cremona	Città di Cremona	NA		
104	NA	Lombardia	Como	Como	NA		
105	NA		Milano	Milano Nosedo	NA		
106	NA		Milano	Milano S. Rocco	NA		
107	NA		Pavia	Pavia	NA		
108	NA		Sondrio	Sondrio	NA		

22	27086	Marche	Pesaro-Urbino	Borgheria	1,88E+04		
23	27087		Pesaro-Urbino	Ponte Metauro	2,69E+04		
24	27088		Ancona	Zipa	5,08E+04		
25	27089		Ancona	Falconara	4,41E+04		
68	27100	Molise	Campobasso	Termoli - località Pantano Basso	<LOD		
69	27099		Campobasso	Termoli - località Porto	4,69E+02		
70	27098		Campobasso	Campobasso - San Pietro	7,65E+02		
49	27131	A.P. Bolzano	Bolzano	IDA Bolzano	2,42E+04	Package A + H146Q + R346T + F456L[b]	Omicron JN.1*
50	27132		Bolzano	IDA Merano	1,66E+04		
51	27133		Bolzano	IDA Termeno	2,55E+04		
26	26990	A.P. Trento	Trento	Trento nord	6,78E+04		
27	26991		Trento	Trento sud	4,39E+04		
28	26992		Trento	Rovereto	4,21E+04	Package A + F456L[a]	Omicron JN.1*
29	26970	Piemonte	Alessandria	Alessandria	3,68E+04		
30	26971		Asti	Asti	1,01E+04		
31	26972		Cuneo	Cuneo	3,53E+04		
32	26922		Torino	Castiglione Torinese	2,00E+04		
33	26923		Biella	Biella Nord	7,25E+02		
34	26924		Biella	Biella Sud	1,12E+03		
35	26925		Novara	Novara	7,50E+02	Package A + E154G + Q183H + K304R + R346T + F456L[c]	Omicron JN.1*
36	26962	Puglia	Bari	Bari Est	<LOD		
37	26963		Bari	Bari Ovest	3,29E+01		
38	26964		Taranto	Taranto Bellavista	2,96E+01		
39	26965		Taranto	Taranto Gennarini	<LOD		
55	27090	Sicilia	Catania	Pantano d'Arci	3,95E+03		
56	27091		Catania	Giarre	1,06E+04		
57	27092		Siracusa	Siracusa	8,14E+03		
76	26959		Trapani	Trapani	2,21E+03		
77	26960		Trapani	Mazara del Vallo	<LOD		

78	26961		Trapani	Marsala	2,32E+03		
79	26996		Ragusa	Modica	2,83E+03	Package A + C361F + F456L[a]	Omicron JN.1*
80	26997		Ragusa	Vittoria	8,18E+03		
81	26998		Ragusa	Ragusa	2,35E+03		
82	26999		Caltanissetta	Gela Macchitella	4,95E+03		
83	27119		Palermo	Acqua dei Corsari	2,49E+04		
84	27120		Palermo	Fondo Verde	1,33E+04		
85	27121		Caltanissetta	Caltanissetta e San Cataldo	3,44E+04		
86	27122		Palermo	Bagheria	6,10E+04		
87	27123		Enna	Enna	2,23E+04		
88	27124		Agrigento	Agrigento	4,65E+04		
89	27105	Toscana	Pisa	Pisa Nord - S. Jacopo	5,45E+01		
90	27108		Lucca	Pontetetto	<LOD		
48	26983	Umbria	Perugia	Perugia - Pian della Genna	5,01E+04		
40	27000	Veneto	Verona	Verona_collettore 1M	2,50E+04		
41	27001		Verona	Verona_collettore 3M	2,90E+04	Package A + F456L[a]	Omicron JN.1*
42	27002		Verona	Verona_collettore 8M	4,80E+04		
43	26966		Treviso	Treviso	2,62E+04		
44	26967		Venezia	Venezia Fusina	3,50E+04	Package A + F456L[a]	Omicron JN.1*
45	26968		Vicenza	Vicenza Casale	2,90E+03		

NA= Not available

[a] 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.

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

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

Table 2. Sanger sequencing results

ID SAMPLES	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
2, 10, 28, 41, 44, 79, 94, 95, 96	■	■	■	■	■	■		■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	**Package A (Omicron JN.1* + F456L)**
13, 49, 98	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	**Package A (Omicron JN.1* + R346T + F456L)**
35	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	**Package A (Omicron JN.1* + Q183H + R346T + F456L)**

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.

Acknowledgements

We thank all of the members of the SARI network ("Sorveglianza Ambientale di SARS-CoV-2 attraverso i Reflui urbani in Italia") for the cooperation in sample collection and processing, data gathering and management, organization and logistic support. The SARI network includes:

- **Abruzzo:** Giuseppe Bucciarelli, Paolo Torlontano (Regione Abruzzo); Giuseppe Aprea, Silvia Scattolini, Vicdalia Aniela Acciari (Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale");
- **Basilicata:** Michele La Bianca (Regione Basilicata); Rosa Anna Cifarelli, Achille Palma, Giovanna La Vecchia e Giuseppe Lauria (Agenzia Regionale per la Protezione dell'Ambiente Basilicata – ARPAB); Rosanna Brienza e Patrizia Montenegro (Acquedotto Lucano-AQL);
- **Campania:** Vincenzo Giordano (Regione Campania); Luigi Cossentino (Arpac - Agenzia Regionale per la Protezione Ambientale in Campania); Francesca Pennino, Annalisa Lombardi (Università degli Studi di Napoli "Federico II");
- **Emilia-Romagna:** Paola Angelini, Lisa Gentili (Regione Emilia – Romagna); Laura De Lellis, Daniele Nasci (HERATech); Giovanni Alborali; Nicoletta Formenti, Flavia Guarneri (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna); Nadia Fontani, Giulia Nani, Gianluca Borlone, Marco Guercio (IREN);
- **Friuli-Venezia Giulia**: Marika Mariuz e Gabriella Trani (Direzione Centrale Salute FVG); Fabrizio Agnoletti, Martina Ustulin, Chiara Targhetta (Istituto Zooprofilattico Sperimentale delle Venezie)
- **Lazio:** Carla Ancona (DEPLAZIO - Dipartimento di Epidemiologia del Servizio Sanitario Regionale - Regione Lazio); Alessandra Barca, Flavia Serio (Regione Lazio); Doriana Antonella Giorgi, Irene Ferrante, Monica Monfrinotti, Silvia Riosa, Valeria Capparuccini (ARPA Lazio - Agenzia Regionale per la Protezione Ambientale del Lazio); Maria Teresa Scicluna, Antonella Cersini (IZSLT - Istituto Zooprofilattico Sperimentale del Lazio e della Toscana); Mariaconcetta Arizzi, Giancarlo Cecchini, Claudio Ottaviano (Acea Elabori);
- **Liguria:** Elena Nicosia (Regione Liguria settore tutela della salute negli ambienti di vita e di lavoro); Nadia Fontani, Giulia Nani, Gianluca Borlone, Marco Guercio (Iren); Elena Grasselli; Giorgia Allaria, Lorenzo Dondero, Francesca Rispo (UNIGE - DISTAV); Alberto Izzotti (UNIGE – DIMES); Rosa Maria Bertolotto, Elena Nicosia, Stefano Rosatto, Marta Bellisomi, Irene Tomesani (ARPAL); Micaela Tiso (MICAMO srl);
- **Lombardia:** Emanuela Ammoni, Danilo Cereda (Regione Lombardia); Marina Nadia Losio, Barbara Bertasi (IZSLER - Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia); Desdemona Oliva, Maria Giovanna Guiso, Fabio Ferrari, Maria Mundo ed Antonino Martines (CAP Holding); Sara Castiglioni, Silvia Schiarea, Giulia Salmoiraghi (Istituto Mario Negri IRCCS); Manuela Antonelli, Arianna Azzellino, Francesca Malpei, Andrea Turolla (POLIMI); Sandro Binda, Pellegrinelli Laura, Valeria Primache (Università degli Studi di Milano, Dipartimento di Scienze Biomediche per la Salute), Clementina Cocuzza, Andrea Franzetti, Rosario Musumeci e Marianna Martinelli (Università di Milano-Bicocca); Giorgio Bertanza (Università di Brescia), Maria Luisa Callegari (Università Cattolica del Sacro Cuore);
- **Marche:** Luigi Bolognini, Fabio Filippetti (Regione Marche); Marta Paniccia', Francesca Ciuti, Sara Briscolini (IZSUM - Istituto Zooprofilattico Sperimentale Umbria Marche); Silvia Magi, Elena Ballarini, Annalisa Grucci (ARPAM);

- **Molise:** Michele Colitti (Regione Molise); Carmen Montanaro (ASReM); Giuseppe Aprea, Silvia Scattolini, Daniela D'Angelantonio, Giacomo Migliorati (Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale"); Maria Grazia Cerroni (Arpa Molise);
- **Piemonte:** Bartolomeo Griglio, Renza Berruti, Mauro Cravero, Angela Costa (Regione Piemonte); Manila Bianchi, Lucia Decastelli; Angelo Romano; Clara Tramuta (IZSTO - Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta SC Sicurezza e Qualità degli Alimenti); Elisabetta Carraro, Cristina Pignata (Dipartimento di Scienze della Sanità Pubblica e Pediatriche, Università di Torino), Silvia Bonetta (Dipartimento di Scienze della Vita e Biologia dei Sistemi), Lisa Richiardi (Dipartimento di Scienze della Sanità Pubblica e Pediatriche, Università di Torino);
- **Puglia:** Nehludoff Albano, Giuseppe Di Vittorio, Onofrio Mongelli (Regione Puglia); Osvalda De Giglio, Francesca Apollonio, Francesco Triggiano (Università degli Studi di Bari Aldo Moro - Dipartimento Interdisciplinare di Medicina); Nicola Ungaro (ARPA Puglia);
- **Sicilia:** Mario Palermo (Regione Sicilia); Carmelo Massimo Maida, Walter Mazzucco (Università degli Studi di Palermo-Dipartimento PROMISE - sezione di Igiene); Simona De Grazia, Giovanni Giammanco (Centro di Riferimento Regionale per la Sorveglianza delle Paralisi Flaccide Acute (PFA) e ambientale della circolazione di poliovirus in Sicilia - AOUP Palermo); Giuseppa Purpari (IZS - Istituto Zooprofilattico Sperimentale della Sicilia); Margherita Ferrante; Antonella Agodi, Martina Barchitta (Università degli Studi di Catania - Dipartimento "G. F. Ingrassia");
- **Toscana:** Piergiuseppe Cala' (Regione Toscana); Annalaura Carducci, Marco Verani, Ileana Federigi, Giulia Lauretani, Sara Muzio, Alessandra Pagani (Laboratorio di Igiene e Virologia Ambientale - Dipartimento di Biologia Università di Pisa);
- **Umbria:** Giovanni Santoro (Regione Umbria), Ermanno Federici, Maya Petricciuolo, Sofia Barigelli (Laboratorio Microbiologia Applicata e Ambientale, DCBB Università di Perugia);
- **Veneto:** Francesca Russo, Gisella Pitter, Vanessa Groppi (Regione Veneto); Franco Rigoli, Marco Zampini (ARPAV - Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto); Tatjana Baldovin, Irene Amoruso (Università di Padova);
- **P.A. Bolzano:** Lorella Zago (P.A. Bolzano); Alberta Stenico, Anna-Maria Prast, Morelli Marco, Dossena Matteo (A.P.P.A. Agenzia provinciale per l'ambiente e la tutela del clima, Laboratorio biologico)
- **P.A. Trento:** Francesco Pizzo; Andrea Anselmo, Elena Mengon (P. A. Trento); Maria Cadonna, Mattia Postinghel (ADEP SGI PAT), Francesca Cutrupi, Paola Foladori, Serena Manara (UNITN – Università di Trento).