Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
13th Report
(Study period: 05 – 09 September 2022)

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Main findings:

- Overall, 167 wastewater samples were collected in the week 5-9 September from 18 Regions and 2 Autonomous Provinces (A.P.).
- Overall, 18 samples collected in 8 Regions/A.P. could be successfully amplified by the long PCR assay targeting the spike region.
- Mutations characteristic of the Omicron variant were detected in 8 Regions/A.P., while no sequences were obtained from the remaining Regions.
- Aminoacid substitutions of sublineages BA.5 and BA.4.6 were detected by Sanger sequencing in 72% and 11% of the sequences, respectively.
- NGS results confirmed the presence of sublineages BA.4/BA.5 in the vast majority of the Regions/A.P.

Introduction

On 17 March 2021, the "EU Commission Recommendation 2021/472 on a common approach to establish a systematic surveillance of SARS-CoV-2 and its variants in wastewaters in the EU", strongly encouraged Member States to put in place, no later than 1 October 2021, national wastewater surveillance systems aimed at the collection of data on SARS-CoV-2 and its variants[1].

Following the above EU Recommendation, the Istituto Superiore di Sanità (ISS) instituted "flash surveys", i.e. periodic (monthly) sampling campaigns to be held in different locations in Italy over the course of a brief period, aimed at assessing the diversity of SARS-CoV-2 in wastewater in the country.

The aim of this report is to summarize the results of the thirteenth national flash survey on SARS-CoV-2 variants in wastewater samples collected in Italy in the week 5-9 September 2022.

Methodology

The survey included 167 sewage samples collected at 162 wastewater treatment plants (WTPs) located in 18 regions and 2 autonomous provinces (A.P.). Details on WTPs enrolled in the Surveillance of SARS-CoV-2 in urban wastewater in Italy can be found at the ISS website[2]. Samples were processed by the laboratories of the SARI network (see Acknowledgements). Viral concentration measurements and nucleic acid extraction were performed according to the protocol "Sorveglianza di SARS-CoV-2 in reflui urbani - Protocollo progetto SARI - rev.3". Purified RNAs were shipped in dry ice to ISS, where samples were sequenced as previously described[3] with some modifications.

A long nested RT-PCR assay (ID_980, ~1600 bps, spanning amino acid residues 58 to 573 of the spike protein) was used to detect multiple key nucleotide changes (deletion and/or amino acid substitutions) distinctive of the Variants of Concern (VoCs) and Variants of Interest (VoIs).

[1] Commission Recommendation (EU) 2021/472 of 17 March 2021 on a common approach to establish a systematic surveillance of SARS-CoV-2 and its variants in wastewaters in the EU. (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32021H0472&qid=1628798981209)

[2] 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)

[3] G La Rosa, P. Mancini, G. Bonanno Ferraro, C. Veneri, M. Iaconelli, L. Lucentini, L. Bonadonna, S. Brusaferro, D. Brandtner, A. Fasanella, L. Pace, A. Parisi, D. Galante, E. Suffredini. Rapid screening for SARS-CoV-2 variants of concern in clinical and environmental samples using nested RT-PCR assays targeting key mutations of the spike protein, Water Research, 2021, Volume 197, 1 June 2021, 117104. https://doi.org/10.1016/j.watres.2021.117104.

The amplicons from the long nested assay were sequenced by both Sanger and Next Generation Sequencing (NGS), using the Oxford Nanopore Technology MinION platform. For NGS, the amplicons obtained from different samples collected in the same Region were mixed in a single pool. Bioinformatics analysis of NGS data was carried out as described in La Rosa et al., 2021[4]. Variant calling was performed for the currently recognized VoCs[5].

Since the Omicron sublineages BA.4 and BA.5 have identical spike proteins in the sequenced region, a nested PCR targeting the M gene was also carried out to discriminate between the two Omicron sublineages. Specifically, ORF6-D3N mutation is distinctive of the Omicron BA.5 sublineage.

Results

Overall, 143 of the 167 samples (85.6%) tested positive for SARS-CoV-2 by the real-time RT-qPCR adopted for SARS-CoV-2 environmental surveillance (**Table 1**), viral concentrations ranging from 1.1 E-01 to 3.0E+05 genome copies (g.c.)/L of sewage.

Sanger Sequencing

Real-time PCR, long nested PCR, and sequencing results are summarized in **Tables 1**. Overall, 18 samples from 8 Regions/A.P. (Emilia Romagna, Liguria, Lombardia, A.P. Bolzano, A.P. Trento, Piemonte, Puglia, and Veneto) were amplified by the long PCR assay. All PCR amplicons were characterized as Omicron variant by Sanger sequencing. Specifically, amino acid substitutions of the sublineage Omicron **BA.4/5** L452R and F486V (in addition to those present also in BA.2) were detected in 16 samples (88.8%). Two samples collected in the Regions of Liguria and Puglia also showed the presence of the aminoacid substitution R346T, which - together with L452R and F486V - is suggestive of the presence of sublineage BA.4.6.. Using the assay targeting the M gene, 13 of 16 samples were assigned to Omicron BA.5 (presence of mutation D3N), while no amplicons were obtained for the other three samples.

[4] G La Rosa, D. Brandtner, P. Mancini, C. Veneri, G. Bonanno Ferraro, L. Bonadonna, L. Lucentini, E. Suffredini. 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(18), 2503; https://doi.org/10.3390/w13182503

[5] SARS-CoV-2 variants of concern as of 9 June 2022. SARS-CoV-2 variants of concern as of 9 June 2022 (europa.eu)

Next Generation Sequencing

NGS results could be successfully obtained for all the 8 tested pools, all of which showed characteristic mutations of the Omicron subvariant BA.4/5. Key mutations of sublineage Omicron BA.4.6 (R346T combined with L452R and F486V) were detected in three regions (Liguria and Puglia –already detected by Sanger sequencing – and the A.P of Bolzano).

Sequencing results are summarized in **Table 1**. To improve the readability of the table, mutations were combined into panels ('mutation packages') as follow:

- **Package A** (Omicron BA.4/5, long fragment from PCR ID_980) = **DEL69/70**, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **L452R**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H);

- **Package B** (Omicron BA.4.6, long fragment from PCR ID_980) = **DEL69/70**, G142D, V213G, G339D, **R346T**, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **L452R**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H).

Table 1. PCR and sequencing results (long PCR ID 980)

	Sample ID	Region/A.P.	City	WTP	RT-qPCR (c.g./L)	Mutations found by Sanger sequencing (long PCR ID_980)	SARS-CoV-2 variant (Sanger sequencing)	Sequencing results (NGS)	SARS-CoV-2 variant (NGS)
1	13737	Abruzzo	Chieti	S. Martino	< LOD				
2	13738		Pescara	Villa Carmine	1,20E-01				
3	13739		Pescara	Via Raiale	1,10E-01				
4	13740		L'Aquila	Pile	< LOD				
5	13741		Teramo	Villa Pavone	< LOD				
6	13753	Basilicata	Potenza	Tiera di Vaglio	1,38E+03				
7	13754		Matera	Pantano	7,88E+02				
8	13849	Calabria	Cosenza	Cosenza - Code di volpe	1,43E+03				
9	13850		Cosenza	Cosenza - Sant'Angelo	1,02E+03				
10	13852		Cosenza	Cosenza - Code di volpe	1,74E+03				
11	13853		Reggio Calabria	Ravagnese - località Aeroporto	1,33E+03				
12	13855		Crotone	Crotone - località Papaniciaro	8,47E+02				
13	13856		Catanzaro	Catanzaro Lido - Loc. Verghello	8,90E+02				
165	14082	Campania	Salerno	Eboli	5,65E+03				
166	14083		Salerno	Nocera Sup	< LOD				
167	14084		Salerno	Salerno	3,75E+03				
168	14085		Napoli	Napoli OVEST - Ingresso Principale	< LOD				
169	14086		Napoli	Napoli OVEST - ex ingresso Camaldoli	4,25E+03				
170	14088		Napoli	Area Nolana	< LOD				
171	14090		Napoli	Napoli EST	< LOD				
172	14092		Caserta	Area Casertana	< LOD				
173	14094		Caserta	Villa Literno	< LOD				

14	13636	Emilia Romagna	Modena	Carpi	< LOD			Package A	Omicron BA.4/5
15	13640		Bologna	IDAR	7,08E+03				
16	13641		Modena	Naviglio	2,14E+03	Package A	Omicron BA.4/5[a]		
17	13642		Ravenna	Faenza	< LOD				
18	13643		Bologna	Imola	2,10E+03	Package A	Omicron BA.4/5		
19	13644		Ravenna - Forlì-Cesena	Ravenna	< LOD				
20	13666		Piacenza	Borgoforte	< LOD				
21	13667		Parma	Parma ovest	1,31E+04				
22	13669		Reggio Emilia	Mancasale	5,85E+03				
23	13777		Forlì-Cesena	Forlì	6,70E+04				
24	13778		Forlì-Cesena	Cesena	1,12E+05				
25	13781		Rimini - Forlì-Cesena	S. Giustina	6,17E+04				
28	13788	Friuli Venezia Giulia	Udine	Trieste	1,67E+04				
29	13790		Trieste	Trieste	7,91E+04				
30	14123		Pordenone	Trieste	7,98E+04				
31	13590	Lazio	Viterbo	Viterbo - Strada Bagni	4,23E+03				
32	13591		Latina	Latina Loc Latina Est	7,40E+02				
33	13592		Roma	Anzio - Colle Cocchino	1,35E+03				
34	13593		Latina	Aprilia (Via del Campo)	1,57E+03				
35	13594		Roma	Guidonia - Ponte Lucano	4,13E+03				
36	13595		Roma	Pomezia - Via Cincinnato	7,53E+03				
37	13596		Roma	Velletri (LA CHIUSA-SORBO)	2,41E+03				
38	13715		Roma	Roma Est (linea 1 + linea 2)	7,27E+03				
39	13722		Roma	Roma Nord	5,04E+03				
40	13723		Roma	Roma Sud	4,99E+02				
41	13724		Roma	Ostia	4,91E+03				
42	13725		Roma	Fregene	8,13E+03				
164	13830		Roma	Civitavecchia Fiumaretta	2,50E+01				

43	13663		Genova	Punta Vagno Genova	1,04E+04			
44	13917		Savona	Savona	9,49E+04			
45	13918		Genova	Pegli	1,63E+05			
46	13919		Genova	Voltri	8,54E+04			
47	13920		Genova	Quinto	2,48E+05			
48	13921		Genova	Rapallo	1,38E+05			
49	13922		Genova	Sestri P	3,19E+04			
50	13923		Genova	Sturla	9,94E+04			
51	13924	Liguria	Savona	Borghetto Santo Spirito	5,56E+04		• Package A • Package B	• Omicron BA.4/5 • Omicron BA.4.6
52	13925		Imperia	Imperia	9,24E+04			
53	13926		Imperia	Sanremo - località Capo Verde	4,84E+04			
54	13927		La Spezia	Camisano	2,99E+04			
55	13928		La Spezia	Silea	1,82E+05			
56	13929		La Spezia	La Spezia	2,99E+05	Package B	Omicron BA.4.6	
57	13930		Genova	Darsena	5,87E+04			
58	13931		Genova	Punta Vagno Genova	5,31E+04			
59	13932		Genova	Valpolcevera	3,59E+04	Package A	Omicron BA.4/5	
60	13597		Milano	Bresso	1,31E+04			
61	13598		Milano - Monza e della Brianza	Peschiera Borromeo	2,58E+04			
62	13599		Milano - Varese	Canegrate	3,70E+04	Package A	Omicron BA.4/5	
63	13600		Varese	Varese	6,10E+04			
64	13601		Milano - Varese	Lonate Pozzolo	7,45E+04			
65	13657	Lombardia	Milano	Milano Nosedo	4,12E+04		Package A	Omicron BA.4/5
66	13658		Milano	Milano San Rocco	9,76E+04			
67	13659		Como	Como	5,07E+04			
68	13660		Pavia	Pavia	2,71E+04			
69	13661		Como - Lecco - Milano - Monza e della Brianza	Monza	< LOD			

70	13662		Pavia	Vigevano	4,27E+04				
71	13721		Sondrio	Sondrio	1,11E+05	Package A	Omicron BA.4/5[a]		
72	13748		Bergamo	Bergamo	< LOD				
73	13749		Cremona	Citta di Cremona	9,52E+02				
74	13751		Brescia	Verziano	2,22E+03				
75	13726	Marche	Pesaro-Urbino	Borgheria	1,97E+03				
76	13727		Pesaro-Urbino	Ponte Metauro	1,19E+03				
77	13728		Pesaro-Urbino	Ponte Sasso	1,78E+03				
78	13729		Ancona	Zipa	4,57E+03				
79	13730		Ancona	Falconara	6,95E+03				
80	13731		Ancona	Camerano	8,23E+03				
81	13745	Molise	Campobasso	Campobasso - San Pietro	< LOD				
82	13746		Campobasso	Termoli - località Porto	< LOD				
83	13747		Campobasso	Termoli - località Pantano Basso	6,00E-02				
84	13633	P.A. Bolzano	Bolzano	IDA Bolzano	5,14E+04			• Package A • Package B	• Omicron BA.4/5 • Omicron BA.4.6
85	13634		Bolzano	IDA Merano	6,78E+04				
86	13635		Bolzano	IDA Termeno	8,52E+04	Package A	Omicron BA.4/5[a]		
87	13573	P.A. Trento	Trento	Trento nord	9,20E+04	Package A	Omicron BA.4/5[a]	Package A	Omicron BA.4/5
88	13574		Trento	Trento sud	4,42E+04				
89	13575		Trento	Rovereto	1,83E+05				
90	13553	Piemonte	Torino	Castiglione Torinese	3,50E+04			Package A	Omicron BA.4/5
91	13554		Novara	Novara	9,28E+03				
92	13555		Biella	Biella Nord	3,29E+04	Package A	Omicron BA.4/5[a]		
93	13556		Biella	Biella Sud	2,30E+04				
94	13622		Alessandria	Alessandria	9,25E+02				
95	13624		Asti	Asti	1,03E+03				
96	13625		Cuneo	Cuneo	1,68E+03				
97	13527	Puglia	Bari	Bari Est	6,16E+02			• Package A • Package B	• Omicron BA.4/5
98	13528		Bari	Bari Ovest	6,72E+02				

99	13529		Brindisi	Brindisi Fiume Grande	1,18E+03			• Omicron BA.4.6
100	13530		Lecce	Lecce	1,09E+03			
101	13531		Taranto	Taranto Gennarini	1,30E+03			
102	13532		Taranto	Taranto Bellavista	2,77E+02	Package A	Omicron BA.4/5[a]	
103	13585		Barletta-Andria-Trani	Andria	1,49E+03			
104	13586		Barletta-Andria-Trani	Barletta	1,39E+03			
105	13587		Foggia	Manfredonia	2,18E+02			
106	13588		Foggia	Cerignola	1,88E+02	Package B	Omicron BA.4.6	
107	13589		Foggia	Foggia	1,34E+03			
108	13611		Bari	Bitonto	1,57E+02			
109	13612		Barletta-Andria-Trani	Bisceglie	9,89E+02	Package A	Omicron BA.4/5[a]	
110	13613		Bari	Molfetta	4,32E+01			
111	13614		Barletta-Andria-Trani	Trani	5,67E+02			
112	13615		Bari	Altamura	1,62E+03			
113	13602		Trapani	Trapani	7,78E+03			
114	13603		Trapani	Mazara del Vallo	4,80E+03			
115	13604		Trapani	Marsala	2,06E+04			
116	13757		Messina	Mili Marina	6,00E+02			
117	13758		Caltanissetta	Gela Macchitella	6,38E+02			
118	13759		Messina	Mili Marina	6,50E+02			
119	13760	Sicilia	Ragusa	Ragusa	5,88E+02			
120	13773		Catania	Pantano d'Arci	-			
121	13775		Catania	Giarre	-			
122	13776		Siracusa	Siracusa	-			
123	13820		Agrigento	Agrigento	2,46E+03			
124	13821		Enna	Enna	1,90E+04			
125	13822		Palermo	Bagheria	8,01E+03			

126	13823		Palermo	Acqua dei Corsari	7,10E+03				
127	13824		Palermo	Fondo Verde	9,99E+03				
128	13825		Caltanissetta	Caltanissetta e San Cataldo	1,57E+04				
133	13761	Toscana	Pisa	Pisa Nord - S. Jacopo	2,12E+04				
134	13762		Firenze	Empoli Pagnana	< LOD				
135	13763		Massa	Lavello 2	< LOD				
136	13764		Lucca	Viareggio	2,07E+04				
137	13765		Massa	Lavello 1	1,47E+04				
138	13766		Lucca	Pontetetto	5,27E+04				
139	13767		Livorno	Rivellino	< LOD				
140	13768		Livorno	Rivellino	1,65E+04				
141	13808		Firenze	San Colombano	5,10E+03				
142	13809		Firenze	San Colombano	7,58E+03				
143	13810		Prato	Baciacavallo	2,73E+04				
144	13811		Prato	Baciacavallo	4,23E+04				
145	13813		Arezzo	Casolino - San Leo	2,08E+04				
146	13814		Grosseto	San Giovanni - Pianetto	1,86E+04				
147	13815		Siena	Ponte a Tressa	< LOD				
148	13817		Pistoia	Centrale Pistoia	9,25E+03				
149	13654	Umbria	Perugia	Perugia - Pian della Genna	3,37E+04				
150	13655		Perugia	Foligno Casone	3,54E+03				
151	13656		Terni	Terni	1,72E+04				
152	13789	Valle D'Aosta	Aosta	La Salle	2,40E+03				
153	13793		Aosta	Brissogne	6,99E+03				
154	13557	Veneto	Padova	Padova Ca' Nordio - centro storico	2,94E+04	Package A	Omicron BA.4/5[a]	Package A	Omicron BA.4/5
155	13558		Padova	Padova Ca' Nordio - zip	2,83E+04				
156	13559		Padova	Padova Guizza	2,90E+04				
157	13560		Padova	Abano Terme	6,27E+04	Package A	Omicron BA.4/5[a]		
158	13608		Treviso	Treviso	3,05E+03	Package A	Omicron BA.4/5[a]		

159	13609	Venezia	Venezia Fusina	5,73E+03	Package A	Omicron BA.4/5[a]
160	13610	Vicenza	Vicenza Casale	2,98E+03		
161	13645	Verona	Verona_collettore 1M	1,88E+03		
162	13646	Verona	Verona_collettore 3M	2,93E+03	Package A	Omicron BA.4/5[a]
163	13647	Verona	Verona_collettore 8M	1,68E+03	Package A (partial, from G339D to Y505H)[b]	Omicron BA.4/5[a]

[a] the presence of mutation D3N in the M gene is suggestive of the presence of BA.5

[b] partial sequence due to mixed electropherograms and/or high signal noise; within brackets the region for which a sequence was provided.

- **Package A** (Omicron BA.4/5, long fragment from PCR ID_980) = **DEL69/70**, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **L452R**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H);
- **Package B** (Omicron BA.4.6, long fragment from PCR ID_980) = **DEL69/70**, G142D, V213G, G339D, **R346T**, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **L452R**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H).

Table 2. Sanger sequencing results (long PCR ID 980)

	CHARACTERISTIC MUTATIONS																					VARIANTS
ID SAMPLES	DEL69/70	G142D	V213G	G339D	R346T	S371F	S373P	S375F	T376A	D405N	R408S	K417N	N440K	L452R	S477N	T478K	E484A	F486V	Q493R	Q498R	N501Y	Y505H	
16-18-59-62-71-86-87-92-102-109-154-157-158-159-162-163																							**Package A*** (Omicron BA.4/5)
56-106																							**Package B** (Omicron BA.4.6)

* Upon testing for the assay targeting the M gene, 13 of the 16 BA.4/BA.5 sequences were assigned to Omicron BA.5.

Limitations of the study

This flash survey's geographical and population coverage was incomplete, as it covered 20/21 of the Italian regions/Autonomous Provinces.
Molecular analytical methods applied to complex environmental matrices as wastewaters may be hampered by low viral concentration, poor recovery of the analyte, and/or inhibition of PCR amplification. Therefore, both detection/quantification and PCR amplification for sequencing purposes may produce false negatives. Consequently, molecular characterization and variant detection may not be achieved for all samples.
Partial sequencing of the Spike region does not allow conclusive assignation of sublineages. However, the detection within the Spike region of multiple, linked mutations associated to specific lineages/sublineages is strongly suggestive of their presence. Therefore, the detection, either by Sanger or NGS sequencing, of defined mutation panels characteristic of certain lineages/sublineages should be considered as a presumptive detection.

Conclusions and final considerations

This is the thirteenth of a series of monthly reports on SARS-CoV-2 and its variants in wastewaters that will continue to be issued as a part of the surveillance established in Italy under EU Commission Recommendation 2021/472, with the aim of providing information on SARS-CoV-2 variants in the population to supplement information acquired through the clinical surveillance.
The results of SARS-CoV-2 surveillance in wastewaters showed the exclusive presence of the omicron variant in Italy, with prevalence of sublineage BA.5. Mutations characteristic of Omicron sublineage BA.4.6 were also detected in 11% of sequenced samples.

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