Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
16th Report
(Study period: November 27th to December 2nd, 2022)

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Main findings:

- Between November 27th and December 2nd, 2022, a total of 171 wastewater samples were collected from 18 Regions and 2 Autonomous Provinces.
- The characteristic mutations of the Omicron variant were detected in 18 Regions/Autonomous Provinces, while no sequences were obtained from the remaining regions.
- Of the sequences obtained by Sanger sequencing, 58.1% showed amino acid substitutions of sublineage BQ.1.1, 16.3% of BA.4/5 + R346T, 11.6 % of BA.4/5, 4.6% of BQ.1 and 2.3% of BA.2.75.
- Next-generation sequencing (NGS) results confirmed the widespread presence of sublineages BQ.1.1 across the majority of the Regions/Autonomous Provinces and the circulation of sublineages BA.4/5, BA.4/5 + R346T, BQ.1, and BA.2.75. Additionally, NGS detected XBB.1 in three Regions.

Introduction

On March 17th, 2021, the European Union Commission issued Recommendation 2021/472, which encouraged 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) initiated "flash surveys", which involve periodic, monthly sampling campaigns conducted at various locations throughout Italy over a short period of time. The purpose of this report is to provide a summary of the findings from the 16th national flash survey on SARS-CoV-2 variants in wastewater samples in Italy, which was conducted from November 27th to December 2nd, 2022.

Methodology

The survey conducted from November 27th to December 2nd, 2022 involved the collection of 171 sewage samples from 167 wastewater treatment plants (WTPs) located across 18 Regions and 2 Autonomous Provinces. Information on the WTPs participating in the SARS-CoV-2 surveillance in urban wastewater in Italy is available on the ISS website[1]. The samples were processed and the viral concentration was determined by the SARI network laboratories using the protocol “Sorveglianza di SARS-CoV-2 in reflui urbani - Protocollo progetto SARI - rev.3”[2]. The purified RNAs were then sent to ISS for variant detection, using both Sanger and NGS methods.

To screen for the presence of the Omicron variant, a real-time RT-PCR assay was used[3]. Additonally, a long nested RT-PCR assay spanning amino acid residues 58 to 573 of the spike protein (~1600 bps),) was used to detect multiple nucleotide changes distinctive of Variants of Concern (VoCs) and Variants of Interest (VoIs) in the spike protein[4]. Amplicons from the long nested assay were sequenced using both Sanger (single samples) and Next Generation Sequencing (NGS) (pools by Regions/AP) with the

[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] La Rosa G, Iaconelli M, Veneri C, Mancini P, Bonanno Ferraro G, Brandtner D, Lucentini L, Bonadonna L, Rossi M, Grigioni M; SARI network; Suffredini E. The rapid spread of SARS-COV-2 Omicron variant in Italy reflected early through wastewater surveillance. Sci Total Environ. 2022 Sep 1;837:155767. doi: 10.1016/j.scitotenv.2022.155767. Epub 2022 May 6. PMID: 35533857; PMCID: PMC9074219.

[4] 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.

Oxford Nanopore Technology MinION platform. Bioinformatics analysis was carried out. and variant calling was performed for recognized VoCs, as previously described[5].

Results

Out of 171 samples collected, 158 (92.4%) tested positive for SARS-CoV-2 using the real-time RT-qPCR method used for environmental surveillance (Table 1). The viral concentrations ranged from 1.00E+01 to 3.30E+06 genome copies (g.c.) per liter of sewage. Additionally, 164 out of the 171 samples (96%) tested positive for the Omicron variant, using the RT-qPCR assay, with cycle threshold (CT) values ranging from 30.8 to 39.9.

Sanger Sequencing

Table 1 summarizes the results of the real-time PCR assays, long nested PCR, and sequencing . A total of 47 samples from 18 Regions/Autonomous provinces were amplified by the long nested PCR assay. Long amplicons could not be obtained from samples collected in the regions of Basilicata and Calabria. Sanger sequencing yielded high-quality sequences from 43 samples, while four sequences were unsuccessful due to high background or noisy sequencing signal.. All amplicons sequenced through Sanger sequencing were characterized as the Omicron variant

Specifically, the Omicron sublineage BA.4/5 was detected in 5 samples (11.6%) from 5 Regions (Friuli-Venezia Giulia, Lazio, Toscana, Umbria, and Veneto), while sublineage BA.4/5 + R346T was detected in 7samples (16.3%) from 6 Region and one AP (Emilia Romagna, Friuli-Venezia Giulia, Liguria, Piemonte, Veneto, and A.P. of Trento). Sublineage BQ.1 was detected in 4.6% of the samples, and BQ.1.1 was detected in 58.1% of the samples. Three samples (7%) showed the presence of double peaks in correspondence to the spike mutation sites 1038, 1332, and 1380 (corresponding to the aminoacids R346, K444, and N460, respectively) suggesting the simultaneous presence of more than one sublineage in different combinations (BA.4/5, BA.4/5 + R346T, BQ.1, or BQ.1.1). Only one sample (2.3%) presented the key mutations of Omicron BA.2.75.

Next Generation Sequencing

NGS results were successfully obtained for all tested Regional pools, which showed the defining mutations of the Omicron variant (Table 1).
For ease of reading, the mutations were grouped into panels ('mutation packages') as follow:

- **Package A** (Omicron BA.4/5) = 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.4/5 + R346T) = DEL69/70, G142D, V213G, G339D, **R346T**, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **L452R**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H
- **Package C** (Omicron BQ.1) = DEL69/70, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **K444T, L452R**, **N460K,** S477N, T478K, E484A, **F486V,** Q498R, N501Y, Y505H

[5] 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

- **Package D** (Omicron BQ.1.1) = DEL69/70, G142D, V213G, G339D, **R346T**, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, **K444T**, **L452R**, **N460K**, S477N, T478K, E484A, **F486V**, Q498R, N501Y, Y505H
- **Package E** (Omicron XBB.1) = V83A, G142D, DEL144, H146Q, Q183E, V213E, G252V, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K, S477N, T478K, E484A, F486S, F490S, Q498R, N501Y, Y505H
- **Package F** (Omicron BA.2.75) = G142D, K147E, W152R, F157L, I210V, V213G, G257S, G339H, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, G446S, N460K, S477N, T478K, E484A, Q498R, N501Y, Y505H

The key mutations of the Omicron sublineage BA.4/5 (Package A) were detected in 10 Regions of Italy, namely Campania, Friuli-Venezia Giulia, Lazio, Liguria, Lombardia, Molise, Toscana, Umbria, Valle d'Aosta and Veneto. The key mutations of the Omicron sublineage BA.4/5 + R346T (Package B) were detected in eleven Regions/AP: Campania, Emilia Romagna, Friuli-Venezia Giulia, Lazio, Liguria, Lombardia, Molise, Piemonte, Valle d'Aosta, Veneto, A.P of Trento. The amino-acid substitutions of the sublineage BQ.1 (Package C) were found in eight Regions: Friuli-Venezia Giulia, Lazio, Liguria, Lombardia, Molise, Puglia, Valle d'Aosta and Veneto. The sublineage BQ.1.1 (Package D) was detected in most of Regions/A.P except for Umbria. In addition, sublineage XBB.1 was detected in three Regions (Friuli Venezia Giulia, Lazio, and Veneto), while sublineage BA.2.75 was detected in three other Regions (Emilia Romagna, Liguria, and Veneto).

Table 1. PCR and sequencing results

	Sample ID	Region/A.P.	City	WTP	RT-qPCR (c.g./L)	RT-qPCR Omicron-ID 999 (CT value)	Mutations found by Sanger sequencing (long PCR ID_980)	SARS-CoV-2 variant (Sanger sequencing)	Sequencing results (NGS)	SARS-CoV-2 variant (NGS)
18	16361	Abruzzo	Chieti	S. Martino	2,32E+03	38.04			• **Package D + V83A + Y144del**	• Omicron BQ.1.1 + V83A + Y144del
19	16362		Pescara	Via Raiale	6,48E+03	35.74				
20	16363		Pescara	Villa Carmine	1,52E+03	38.05				
21	16364		L'Aquila	Pile	4,32E+03	36.77	**Package D + V83A + DEL144/144**	Omicron BQ.1.1 + V83A + DEL144/144		
22	16365		Teramo	Villa Pavone	2,19E+03	35.31	-			
23	16369	Basilicata	Potenza	Tiera di Vaglio	2,53E+03	38.26				
24	16370		Matera	Pantano	2,30E+03	ND				
25	16330	Calabria	Cosenza	Cosenza - Sant'Angelo	1,76E+03	36.73				
26	16331		Cosenza	Cosenza - Code di volpe	7,40E+02	36.75				
27	16332		Crotone	Crotone - località Papaniciaro	1,85E+03	34.29				
28	16333		Catanzaro	Catanzaro Lido - Loc. Verghello	4,83E+03	33.43				
29	16334		Catanzaro	Catanzaro - Zona industriale	1,17E+03	33.96				
30	16335		Reggio Calabria	Ravagnese - località Aeroporto	4,76E+03	37.05				
31	16461	Campania	Avellino	Manocalzati	< LOD	37.55			• **Package A +R346I** • **Package B** • **Package D**	• Omicron BA.4/5 +R346I • Omicron BA.4/5 + R346T • Omicron BQ.1.1
32	16462		Salerno	Salerno	< LOD	36.15				
33	16463		Salerno	Nocera Sup	< LOD	36.18				
34	16464		Napoli	Napoli OVEST - Ingresso Principale	< LOD	37.18				
35	16465		Napoli	Napoli OVEST - ex ingresso Camaldoli	1,47E+03	36.33				
36	16466		Napoli	Area Nolana	< LOD	37.65				
38	16468		Caserta	Area Casertana	< LOD	35.92				

170	16467		Napoli	Napoli EST	2,10E-01	ND	**Package D**	Omicron BQ.1.1		
171	16458		Salerno	Nocera Superiore	< LOD	34.61				
172	16457		Salerno	Eboli	< LOD	34.88				
173	16456		Caserta	Villa Literno	1,00E+01	37.45				
174	16455		Caserta	Area Casertana	< LOD	38.20				
176	16459		Salerno	Salerno	< LOD	ND				
40	16337	Emilia Romagna	Bologna	IDAR	2,14E+05	34.43	**Package D**	Omicron BQ.1.1	• **Package B** • **Package D** • **Package F**	• Omicron BA.4/5 + R346T • Omicron BQ.1.1 • Omicron BA.2.75
41	16338		Ravenna - Forlì-Cesena	Ravenna	2,90E+04	ND				
42	16339		Modena	Naviglio	9,00E+03	37.63				
43	16340		Ravenna	Faenza	1,08E+04	39.30				
44	16344		Bologna	Imola	7,80E+04	34.02				
45	16232		Ferrara	Ferrara - Linea 1	1,21E+03	39.08				
46	16233		Ferrara	Ferrara - Linea 2	1,42E+03	ND				
47	16234		Modena	Carpi	1,97E+03	39.64				
48	16347		Forlì-Cesena	Forlì	1,67E+05	33.15	**Package B**	Omicron BA.4/5 + R346T		
49	16349		Forlì-Cesena	Cesena	2,24E+05	32.66				
163	16357		S. Giustina	Rimini - Forlì-Cesena	2,27E+01	33.08	**Package D**	Omicron BQ.1.1		
164	16504		Emilia Romagna	Mancasale	7,60E+04	33.98	**Package D**	Omicron BQ.1.1		
166	16501		Emilia Romagna	Borgoforte	1,29E+04	36.33				
167	16502		Emilia Romagna	Parma Ovest	4,73E+05	38.71				
51	16423	Friuli-Venezia Giulia	Pordenone	Cordenons	2,24E+05	32.01	**Package B**	BA.4/5 + R346T	• Package A • Package B • Package C • Package D • Package E	• Omicron BA.4/5 • Omicron BA.4/5 + R346T • Omicron BQ.1 • Omicron BQ.1.1 • Omicron XBB.1
52	16424		Udine	Udine	3,27E+04	34.56				
53	16425		Trieste	Servola	9,38E+04	33.65	**Package A**	Omicron BA.4/5[a]		
151	16260	Lazio	Lazio	ROMA EST	1,17E+05	32.79	**Package D**	Omicron BQ.1.1	• Package A • Package B • Package C	• Omicron BA.4/5 • Omicron BA.4/5 + R346T • Omicron BQ.1
152	16261		Lazio	ROMA NORD	2,49E+05	32.84	**Package D**	Omicron BQ.1.1		
153	16262		Lazio	ROMA SUD	2,40E+04	34.43				

154	16263		Lazio	Ostia	9,12E+04	34.86			• Package D • Package E	• Omicron BQ.1.1 • Omicron XBB.1
155	16264		Lazio	Fregene	2,41E+05	35.33				
156	16162		Lazio	Viterbo_Strada Bagni	3,44E+01	32.74	**Package D**	Omicron BQ.1.1		
157	16163		Lazio	Aprilia Via del Campo	2,27E+01	34.06				
158	16164		Lazio	Anzio Colle Cocchino	2,01E+01	33.63				
159	16165		Lazio	Latina Est	< LOD	ND				
160	16166		Lazio	Pomezia Capoluogo	3,15E+01	33.02				
161	16167		Lazio	La Chiusa - Velletri	3,83E+01	32.68				
162	16168		Lazio	Ponte Lucano di Guidonia	3,70E+01	33.08	**Package A**	Omicron BA.4/5[a]		
169	16398		Lazio	Civitavecchia	1,00E+01	34.18				
54	16273	Liguria	Genova	Voltri	6,40E+05	31.92	**Package D**	Omicron BQ.1.1	• **Package A** • **Package B** • **Package C** • **Package C+ Y144del** • **Package D** • **Package D+ Y144del** • **Package F**	• Omicron BA.4/5 • Omicron BA.4/5 + R346T • Omicron BQ.1 • Omicron BQ.1+ Y144del • Omicron BQ.1.1 • Omicron BQ.1.1+ Y144del • Omicron BA.2.75
55	16274		Genova	Quinto	2,15E+05	34.18				
56	16275		Genova	Rapallo	5,25E+05	33.04				
57	16276		Genova	Sestri P	6,02E+05	31.79				
58	16277		Genova	Sturla	4,33E+05	33.40				
59	16278		Savona	Savona	6,10E+05	32.27	**Package B**	Omicron BA.4/5 + R346T		
60	16279		Savona	Borghetto Santo Spirito	1,48E+05	34.70	**Package C + Y144del(143)**	Omicron BQ.1 + Y144del(143)		
61	16280		La Spezia	Camisano	6,26E+05	32.41				
62	16281		La Spezia	Silea	9,87E+05	31.26	**Double peaks in specific positions[b]**	Omicron BA.4/5[a] - BA.4/5 +R346T - BQ.1-BQ.1.1		
63	16282		La Spezia	La Spezia	5,17E+05	32.69	**Double peaks in specific positions[b]**	Omicron BA.4/5[a] - BA.4/5 +R346T - BQ.1-BQ.1.1		
64	16283		Imperia	Imperia	1,82E+05	34.02				
65	16284		Imperia	Sanremo - località Capo Verde	4,13E+05	37.66				
66	16285		Genova	Darsena	2,05E+05	33.96				
67	16286		Genova	Punta Vagno Genova	4,68E+05	32.97				
68	16287		Genova	Valpolcevera	1,06E+06	30.75	**Package F**	Omicron BA.2.75		

69	16272		Genova	Pegli	2,38E+05	34.19				
165	16498		Liguria	Punta Vagno Genova	4,65E+04	33.02	**Double peaks in specific positions**[c]	Omicron BQ.1-BQ.1.1		
70	16200	Lombardia	Milano	Bresso	2,12E+04	32.81			• **Package A** • **Package B** • **Package C** • **Package D**	• Omicron BA.4/5 • Omicron BA 4/5 + R346T • Omicron BQ.1 • Omicron BQ.1.1
71	16201		Milano - Monza e della Brianza	Peschiera Borromeo	4,45E+03	33.56				
72	16202		Milano - Varese	Canegrate	1,34E+04	34.68				
73	16204		Varese	Varese	1,17E+04	33.91				
74	16205		Milano - Varese	Lonate Pozzolo	1,67E+04	33.25	Sequence failure[d]			
75	16251		Milano	Milano Nosedo	2,80E+05	38.16				
76	16252		Milano	Milano San Rocco	2,87E+05	37.28				
77	16253		Como	Como	1,96E+05	36.06				
78	16254		Pavia	Pavia	1,16E+05	36.57	Sequence failure[d]			
79	16255		Como - Lecco - Milano - Monza e della Brianza	Monza	3,06E+05	37.43				
80	16288		Bergamo	Bergamo	4,74E+04	34.22				
81	16289		Brescia	Verziano	3,51E+04	34.88				
82	16291		Cremona	Citta di Cremona	4,13E+04	34.08				
83	16256		Pavia	Vigevano	3,37E+05	37.97				
12	16265	Marche	Pesaro-Urbino	Borgheria	3,26E+04	36.67			• **Package D**	• Omicron BQ.1.1
13	16266		Pesaro-Urbino	Ponte Metauro	1,02E+04	37.46				
14	16267		Pesaro-Urbino	Ponte Sasso	1,23E+04	39.18				
15	16268		Ancona	Zipa	4,95E+04	34.51	**Package D**	Omicron BQ.1.1		
16	16269		Ancona	Falconara	1,85E+04	36.14				
17	16270		Ancona	Camerano	3,50E+04	35.49				
84	16366	Molise	Campobasso	Campobasso - San Pietro	2,37E+02	37.28			• **Package A** • **Package B** • **Package C** • **Package D**	• Omicron BA.4/5 • Omicron BA.4/5 + R346T • Omicron BQ.1 • Omicron BQ.1.1
85	16367		Campobasso	Termoli - località Porto	5,52E+03	35.20	Sequence failure[d]			
86	16368		Campobasso	Termoli - località Pantano Basso	2,26E+02	38.22	Sequence failure[d]			

87	16197	P.A. Bolzano	Bolzano	IDA Bolzano	3,40E+04	35.20	**Package D + Y144del(143)**	Omicron BQ.1.1 + Y144del(143)	• **Package D** • **Package D + Y144del**	• Omicron BQ.1.1 • Omicron BQ.1.1 + Y144del
88	16198		Bolzano	IDA Merano	5,87E+04	33.45				
89	16199		Bolzano	IDA Termeno	6,61E+04	33.64	**Package D + Y144del(143)**	Omicron BQ.1.1 + Y144del(143)		
90	16170	P.A. Trento	Trento	Trento nord	1,88E+05	34.04			• **Package B** • **Package D**	• Omicron BA.4/5 + R346T • Omicron BQ.1.1
91	16171		Trento	Trento sud	1,05E+05	34.67	**Package B**	Omicron BA.4/5 + R346T		
92	16172		Trento	Rovereto	3,65E+05	33.19				
93	16127	Piemonte	Torino	Castiglione Torinese	3,00E+04	34.85			• **Package B** • **Package D**	• Omicron BA.4/5 + R346T • Omicron BQ.1.1
94	16128		Biella	Biella Nord	2,50E+03	35.39	**Package B**	Omicron BA.4/5 + R346T		
95	16129		Biella	Biella Sud	4,93E+03	34.62	**Package D**	Omicron BQ.1.1		
96	16130		Novara	Novara	6,83E+03	33.95	**Package D**	Omicron BQ.1.1		
97	16211		Alessandria	Alessandria	2,17E+04	35.75				
98	16212		Asti	Asti	1,36E+04	35.49				
99	16213		Cuneo	Cuneo	1,60E+04	34.35				
100	16124	Puglia	Bari	Bari Est	9,97E+03	36.58			• **Package C** • **Package C+ Y144del** • **Package D** • **Package D+ Y144del** • **Package D+G261V**	• Omicron BQ.1 • Omicron BQ.1 + Y144del • Omicron BQ.1.1 • Omicron BQ.1.1 + Y144del • Omicron BQ.1.1 +G261V
101	16126		Bari	Bari Ovest	2,80E+04	35.27				
102	16134		Taranto	Taranto Bellavista	1,87E+04	35.62				
103	16135		Taranto	Taranto Gennarini	1,29E+04	36.93				
104	16136		Lecce	Lecce	2,28E+04	35.51				
105	16137		Brindisi	Brindisi Fiume Grande	2,19E+04	36.67	**Package D** + Y144del(143)	Omicron BQ.1.1 + Y144del(143)		
106	16173		Bari	Altamura	3,27E+04	34.18	**Package D +G261V**	Omicron BQ.1.1 +G261V		
107	16186		Barletta-Andria-Trani	Andria	1,82E+04	36.50				
108	16187		Barletta-Andria-Trani	Barletta	2,03E+04	36.65				
109	16188		Foggia	Cerignola	2,34E+04	35.70				
110	16189		Foggia	Foggia	4,02E+04	35.17				
111	16190		Foggia	Manfredonia	3,43E+04	35.14				

112	16206		Barletta-Andria-Trani	Bisceglie	4,04E+04	33.65	**Package C** + Y144del	Omicron BQ.1 + Y144del		
113	16207		Bari	Bitonto	3,21E+03	37.07				
114	16208		Bari	Molfetta	5,53E+03	37.23				
115	16209		Barletta-Andria-Trani	Trani	2,24E+04	34.47				
116	16301	Sicilia	Agrigento	Agrigento	5,78E+04	35.89			• **Package D+ A222S**	• Omicron BQ.1.1 + A222S
117	16184		Trapani	Trapani	4,01E+03	37.52				
118	16185		Trapani	Mazara del Vallo	2,09E+03	ND				
119	16304		Palermo	Bagheria	6,48E+04	34.98				
120	16305		Palermo	Acqua dei Corsari	1,14E+05	34.29				
121	16306		Palermo	Fondo Verde	5,44E+04	35.66				
122	16315		Messina	Mili Marina	2,19E+04	ND	**Package D + A222S**	Omicron BQ.1.1 + A222S		
123	16325		Catania	Pantano d'Arci	1,74E+04	ND				
124	16314		Caltanissetta	Gela Macchitella	1,08E+03	ND				
125	16307		Caltanissetta	Caltanissetta e San Cataldo	2,29E+05	32.54				
126	16308		Enna	Enna	9,05E+04	35.32				
127	16327		Catania	Giarre	3,85E+03	37.53				
128	16311		Ragusa	Modica	1,60E+03	ND				
129	16312		Ragusa	Vittoria	1,11E+04	38.87				
130	16313		Ragusa	Ragusa	6,90E+03	38.23				
168	16328		Siracusa	Siracusa	< LOD	36.33				
131	16341	Toscana	Pisa	Pisa Nord - S. Jacopo	3,36E+04	35.13	**Package D**	Omicron BQ.1.1	• **Package A** • **Package D** • **Package D+ Y144del**	• Omicron BA.4/5 • Omicron BQ.1.1 • Omicron BQ.1.1 + Y144del
132	16343		Massa	Lavello 2	2,53E+05	34.14				
133	16345		Lucca	Viareggio	1,60E+05	33.96				
134	16348		Massa	Lavello 1	3,91E+04	35.70	**Package A**	Omicron Omicron BA.4/5[a]		
135	16351		Lucca	Pontetetto	1,24E+05	35.38				
136	16352		Livorno	Rivellino	1,71E+05	33.84	**Package D + Y144del(143)**	Omicron BQ.1.1 + Y144del		

137	16374		Prato	Baciacavallo	1,13E+04	ND				
138	16342		Firenze	Empoli Pagnana	6,74E+03	39.88				
139	16353		Livorno	Rivellino	1,70E+05	34.74				
140	16375		Prato	Baciacavallo	1,08E+04	39.41				
141	16376		Arezzo	Casolino - San Leo	2,85E+04	36.68				
142	16377		Grosseto	San Giovanni - Pianetto	3,30E+06	35.97				
143	16379		Siena	Ponte a Tressa	< LOD	ND				
144	16378		Pistoia	Centrale Pistoia	1,51E+06	34.47				
145	16131	Umbria	Perugia	Perugia - Pian della Genna	1,31E+05	34.14			• **Package A+ C291R**	• Omicron BA.4/5+ C291R
146	16237		Perugia	Foligno Casone	3,77E+04	37.08				
147	16238		Terni	Terni	2,54E+05	32.63	**Package A + C291R**	Omicron BA.4/5[a] + C291R		
148	16302	Valle d'Aosta	Aosta	La Salle	3,10E+03	39.53			• **Package A** • **Package B** • **Package C** • **Package D**	• Omicron BA.4/5 • Omicron BA.4/5 + R346T • Omicron BQ.1 • Omicron BQ.1.1
149	16303		Aosta	Brissogne	8,37E+04	33.52	**Package D**	Omicron BQ.1.1		
8	16154	Veneto	Padova	Padova Ca' Nordio - centro storico	1,03E+05	36.31	**Package A**	Omicron BA.4/5[a]	• **Package A** • **Package B** • **Package C** • **Package D** • **Package E** • **Package F**	• Omicron BA.4/5 • Omicron BA.4/5 + R346T • Omicron BQ.1 • Omicron BQ.1.1 • Omicron XBB.1 • Omicron BA.2.75
9	16155		Padova	Padova Ca' Nordio - zip	2,78E+05	35.12	**Package D**	Omicron BQ.1.1		
10	16156		Padova	Padova Guizza	2,49E+05	34.57	**Package B**	Omicron BA.4/5 + R346T		
11	16157		Padova	Abano Terme	2,73E+05	34.55	**Package D**	Omicron BQ.1.1		
5	16191		Vicenza	Vicenza Casale	8,19E+04	32.88				
6	16192		Treviso	Treviso	6,57E+04	32.46	**Package D**	Omicron BQ.1.1		
7	16193		Venezia	Venezia Fusina	9,22E+04	33.13	**Package B**	Omicron BA.4/5 + R346T		
1	16241		Verona	Verona_collettore 1M	5,80E+04	32.28				
2	16242		Verona	Verona_collettore 3M	5,82E+04	32.44				
3	16243		Verona	Verona_collettore 8M	4,43E+04	31.67	**Package D**	Omicron BQ.1.1		
4	16271		Venezia	Venezia Fusina	9,12E+04	32.19	**Package D**	Omicron BQ.1.1		

[a] the presence of a double A/G peak in the triplet encoding the aminoacid in position 3 of the M gene (D3/N3) is suggestive of the simultaneous presence of BA.4 and BA.5

[b] Double peaks in three positions: R346 + K444 + N460

[c] Double peaks in one positions: R346

[d] High Background/Noisy Sequencing Signal

ND not detected

Table 2. Sanger sequencing results

ID SAMPLES	CHARACTERISTIC MUTATIONS																																				VARIANTS
	DEL69/70	G142D	K147E	W152R	F157L	Q183E	I210V	V213G	V213E	G252V	G257S	G339D	G339H	R346T	L368I	S371F	S373P	S375F	T376A	D405N	R408S	K417N	N440K	K444T	V445P	G446S	L452R	N460K	S477N	T478K	E484A	F486V	F486S	F490S	Q498R	N501Y	Y505H	
8-53-147-134-162	■	■						■				■				■	■	■	■	■	■	■	■				■		■	■	■	■			■	■	■	**Package A** (Omicron BA.4/5)
7-10-48-51-59-91-94	■	■						■				■		■		■	■	■	■	■	■	■	■				■		■	■	■	■			■	■	■	**Package B** (Omicron BA.4/5+R346T)
60-112	■	■						■				■				■	■	■	■	■	■	■	■	■			■	■	■	■	■	■			■	■	■	**Package C** (Omicron BQ.1)
3-4-6-9-11-15-21-40-54-87-89-95-96-105-106-122-131-136-149-151-152-156-163-164-170	■	■						■				■		■		■	■	■	■	■	■	■	■	■			■	■	■	■	■	■			■	■	■	**Package D** (Omicron BQ.1.1)
68		■	■	■	■		■	■			■		■			■	■	■	■	■	■	■	■			■		■	■	■	■				■	■	■	**Package F** (Omicron BA.2.75)

Limitations of the study

The flash survey conducted in this study had incomplete geographical and population coverage, as it only covered 20 out of 21 Italian regions and Autonomous Provinces.
The molecular analytical methods used to detect viral RNA in complex environmental matrices, such as wastewater, can be challenging due to low viral concentrations, poor analyte recovery, and/or PCR amplification inhibition. As a result, false negative results can occur in both detection/quantification and PCR amplification for sequencing, making it difficult to achieve molecular characterization and variant detection for all samples.
Partial sequencing of the Spike region does not provide conclusive results for sublineage assignment. However, the detection of defined mutation panels that are characteristic of certain lineages/sublineages, through Sanger or NGS sequencing, should be considered as a presumptive detection.

Conclusions and final considerations

This report is part of a monthly series on SARS-CoV-2 and its variants in wastewaters in Italy, as established by the EU Commission Recommendation 2021/472. The aim is to provide additional information on SARS-CoV-2 variants in the population, supplementing information gathered through clinical surveillance. The findings suggest that the Omicron variant is the only SARS-CoV-2 variant currently present in Italy, with sublineage BQ.1.1 being the most prevalent. Nonetheless, mutations characteristic of other sublineages, including Omicron BA.4/5, BA.4/5 + R346T, BQ.1, XBB.1, and BA.2.75, were also detected..

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