



**Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
12th Report
(Study period: 01– 05 August 2022)**

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Main findings:

- In the week 1-5 of August 2022, 169 wastewater samples were collected from 18 Regions and 2 Autonomous Provinces (A.P.).
- Overall, 29 samples collected in 14 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 all the samples by sequencing (Sanger and NGS)
- Amino acid substitutions of sublineage BA.5 were detected in 85% of the samples by Sanger sequencing, while substitutions of sublineages BA.4 and BA.2 were detected in 7.4% and 3.7% of 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 variants¹.

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 twelfth national flash survey on SARS-CoV-2 variants in wastewater samples collected in Italy in the week of 1-5 August 2022.

Methodology

The survey included 169 sewage samples collected at 163 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 website². 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 described³, 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 \(iss.it\)](https://www.iss.it/sites/default/files/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. Brusaferrò, 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) (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., 2021⁴. Variant calling was performed for the currently recognized VoCs⁵.

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, 155 of the 169 samples (91.7%) 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 5.1 E+01 to 2.3E+06 genome copies (g.c.)/L of sewage.

Sanger Sequencing

Real-time PCR, long nested PCR, and sequencing results are summarized in **Table 1**. Overall, 29 samples from 14 Regions/A.P. were amplified by the long PCR assay. Long amplicons could not be obtained from samples collected in the regions of Abruzzo, Basilicata, Campania, Lombardia, Umbria and Valle d'Aosta. In the remaining Regions, good quality sequences were obtained for 27 samples, whereas – due to the simultaneous presence of more than one strain – mixed electropherograms were obtained for the remaining two samples.

Only mutations characteristic of the Omicron variant were detected by Sanger sequencing. Specifically, amino acid substitutions of the sublineage Omicron **BA.4/5** (L452R and F486V in addition to those present also in Omicron BA.2) were detected in 26 samples (96.3%) from 11 Regions/A.P (Calabria, Emilia Romagna, Lazio, Marche, Molise, A.P. Bolzano, A.P. Trento, Puglia, Sicilia, Toscana, Veneto). One sample also showed the presence of the amino acid 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, 23 samples were assigned to Omicron BA.5 (presence of mutation D3N in the M gene), one to BA.4, and one showed the presence of both subvariants (presence of a double A/G peak in correspondence to the mutation site). Mutation of the Omicron sublineage **BA.2** were detected in one sample from the Region of Piemonte.

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 ECDC. SARS-CoV-2 variants of concern as of 20 October 2022
. [SARS-CoV-2 variants of concern as of 20 October 2022 \(europa.eu\)](https://ecdc.europa.eu/en/sars-cov-2-variants-of-concern)

Next Generation Sequencing

NGS results could be successfully obtained for all the 14 tested pools, all of which showed characteristic mutations of the Omicron variant. Overall, 100% of the reads were assigned to Omicron BA.4/5.

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.2, long fragment from PCR ID_980) = G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, S477N, T478K, E484A, Q493R, Q498R, N501Y, Y505H)
- **Package C** (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	12757	Abruzzo	Chieti	S. Martino	< LOD				
2	12758		Pescara	Via Raiale	6,39E+02				
3	12759		Pescara	Villa Carmine	9,31E+02				
4	12760		L'Aquila	Pile	6,06E+02				
5	12761		Teramo	Villa Pavone	7,72E+02				
6	12752	Basilicata	Potenza	Tiera di Vaglio	1,97E+03				
7	12753		Matera	Pantano	1,58E+03				
8	12746	Calabria	Cosenza	Cosenza - Sant'Angelo	3,58E+03	Package A	Omicron BA.4/5 ^a	Package A	Omicron BA.4/5
9	12747		Cosenza	Cosenza - Code di volpe	1,36E+03				
10	12748		Catanzaro	Catanzaro Lido - Loc. Verghello	1,35E+03				
11	12749		Catanzaro	Catanzaro - Zona industriale	2,78E+03				
12	12750		Crotone	Crotone - località Papaniciaro	4,77E+03				
13	12751		Reggio Calabria	Ravagnese - località Aeroporto	1,28E+03				
164	12982		Campania	Avellino	Manocalzati	3,75E+03			
165	12983	Salerno		Nocera Sup.	3,25E+03				
166	12984	Salerno		Salerno	3,50E+03				
167	12985	Napoli		Napoli OVEST (ingresso principale)	3,80E+03				
168	12986	Napoli		Impianto Napoli OVEST (ingresso camaldoli)	1,15E+04				
169	12987	Napoli		Area Nolana	1,80E+03				
170	12988	Napoli		Napoli EST	3,75E+03				

171	12989		Caserta	Area Casertana	2,08E+03		
172	12990		Caserta	Villa Literno	5,75E+03		
14	12690		Ferrara	Ferrara - Linea 1	3,23E+03	Package A	Omicron BA.4/5
15	12691		Ferrara	Ferrara - Linea 2	< LOD		
16	12692		Modena	Carpi	2,30E+03		
17	12806		Piacenza	Borgoforte	6,28E+02		
18	12807		Parma	Parma ovest	8,73E+03		
19	12809		Reggio Emilia	Mancasale	8,48E+03		
20	12858		Bologna	IDAR	2,33E+04		
21	12859	Emilia Romagna	Modena	Naviglio	6,70E+03	Package A	Omicron BA.4/5 ^a
22	12860		Ravenna - Forlì-Cesena	Ravenna	1,23E+03		
23	12861		Ravenna	Faenza	1,57E+04		
24	12862		Bologna	Imola	1,30E+04		
25	12863		Forlì-Cesena	Cesena	4,73E+04		
26	12865		Forlì-Cesena	Forlì	1,66E+04		
27	12869		Rimini - Forlì- Cesena	S. Giustina	1,88E+04		
28	13448		Friuli Venezia Giulia	Pordenone	Cordenons	3,75E+02	Package A
29	13449	Udine		Udine	1,30E+04	Package C	Omicron BA.4.6
30	13452	Trieste		Servola	5,24E+04		
31	12631		Viterbo	Viterbo - Strada Bagni	3,53E+03	Package A	Omicron BA.4/5
32	12632		Latina	Latina Loc Latina Est	3,43E+03	Package A	Omicron BA.4/5 ^a
33	12633		Roma	Anzio - Colle Cocchino	2,38E+03		
34	12634		Latina	Aprilia (Via del Campo)	1,08E+04		
35	12635	Lazio	Roma	Velletri (LA CHIUSA-SORBO)	1,74E+03	Package A	Omicron BA.4/5 ^b
36	12636		Roma	Pomezia - Via Cincinnato	1,76E+04		
37	12680		Roma	Civitavecchia Fiumaretta	2,50E+02		
38	12671		Roma	Roma Est (linea 1 + linea 2)	3,83E+04		
39	12772		Roma	Roma Nord	3,73E+04		

40	12773		Roma	Roma Sud	9,67E+03		
41	12774		Roma	Ostia	2,14E+04		
42	12775		Roma	Fregene	2,67E+04		
43	12898		Savona	Savona	6,16E+04	Package A	Omicron BA.4/5
44	12899		Savona	Borghetto Santo Spirito	9,32E+04		
45	12900		Genova	Pegli	2,67E+05		
46	12901		Genova	Voltri	9,20E+04		
47	12902		Genova	Quinto	7,48E+04		
48	12903		Genova	Rapallo	8,63E+04		
49	12904		Genova	Sestri P	6,07E+04		
50	12905		Genova	Sturla	2,47E+04		
51	12906	Liguria	La Spezia	Camisano	4,85E+05		
52	12907		La Spezia	Silea	3,66E+05		
53	12908		La Spezia	La Spezia	6,65E+05		
54	12909		Imperia	Imperia	2,28E+05		
55	12910		Imperia	Sanremo - località Capo Verde	2,77E+05		
56	12911		Genova	Darsena	7,38E+04		
57	12912		Genova	Punta Vagno Genova	1,85E+05		
58	12913		Genova	Valpolcevera	1,43E+05		
59	12803		Genova	Punta Vagno Genova	2,75E+04	ME ^c	
60	12655		Milano	Bresso	2,07E+04		
61	12656		Milano - Monza e della Brianza	Peschiera Borromeo	8,40E+03		
62	12657	Lombardia	Milano - Varese	Canegrate	7,08E+04		
63	12658		Varese	Varese	3,68E+04		
64	12659		Milano - Varese	Lonate Pozzolo	4,70E+04		
65	12762		Bergamo	Bergamo	1,31E+04		
66	12763		Cremona	Citta di Cremona	5,33E+03		

67	12765		Brescia	Verziano	2,65E+03		
68	13495		Sondrio	Sondrio	1,87E+04		
69	13505		Milano	Milano Nosedo	< LOD		
70	13506		Milano	Milano San Rocco	1,00E+05		
71	13507		Como	Como	7,38E+04		
72	13508		Pavia	Pavia	1,27E+05		
73	13509		Como - Lecco - Milano - Monza e della Brianza	Monza	1,01E+05		
74	13511		Pavia	Vigevano	2,16E+04		
75	12699	Marche	Pesaro-Urbino	Borgheria	1,27E+04		Package A Omicron BA.4/5
76	12700		Pesaro-Urbino	Ponte Metauro	3,19E+03		
77	12701		Pesaro-Urbino	Ponte Sasso	8,39E+03		
78	12702		Ancona	Zipa	3,49E+04	Package A	Omicron BA.4/5 ^d
79	12703		Ancona	Falconara	1,93E+04		
80	12704		Ancona	Camerano	1,74E+04		
81	12754	Molise	Campobasso	Campobasso - San Pietro	< LOD		Package A Omicron BA.4/5
82	12755		Campobasso	Termoli - località Porto	6,39E+02		
83	12756		Campobasso	Termoli - località Pantano Basso	1,62E+03	Package A	Omicron BA.4/5 ^a
161	12844	P.A. Bolzano	Bolzano	IDA Bolzano	3,56E+04		Package A Omicron BA.4/5
162	12845		Bolzano	IDA Merano	1,96E+04	Package A	Omicron BA.4/5 ^a
163	12847		Bolzano	IDA Termeno	3,07E+04	Package A	Omicron BA.4/5 ^a
84	12649	P.A. Trento	Trento	Trento nord	1,98E+05	Package A	Omicron BA.4/5 ^a Package A Omicron BA.4/5
85	12650		Trento	Trento sud	9,04E+04		
86	12651		Trento	Rovereto	2,57E+05		
87	12627	Piemonte	Torino	Castiglione Torinese	6,98E+02		Package A Omicron BA.4/5
88	12628		Biella	Biella Nord	9,25E+02		
89	12629		Biella	Biella Sud	5,12E+01	Package B	Omicron BA.2

90	12630		Novara	Novara	1,70E+03			
91	12674		Alessandria	Alessandria	2,93E+03			
92	12675		Asti	Asti	1,70E+03			
93	12676		Cuneo	Cuneo	7,35E+03			
94	12616	Puglia	Bari	Bari Est	2,29E+03	Package A	Omicron BA.4/5 ^a	Package A Omicron BA.4/5
95	12617		Bari	Bari Ovest	4,08E+03			
96	12637		Lecce	Lecce	6,18E+03			
97	12638		Brindisi	Brindisi Fiume Grande	3,16E+03			
98	12639		Taranto	Taranto Bellavista	5,85E+03	Package A	Omicron BA.4/5 ^a	
99	12640		Taranto	Taranto Gennarini	4,21E+03	Package A	Omicron BA.4/5 ^a	
100	12652		Bari	Altamura	3,16E+03	Package A	Omicron BA.4/5 ^a	
101	12660		Foggia	Cerignola	4,06E+03	Package A	Omicron BA.4/5 ^a	
102	12661		Foggia	Manfredonia	5,02E+03	Package A	Omicron BA.4/5 ^a	
103	12662		Foggia	Foggia	2,30E+03	Package A	Omicron BA.4/5 ^a	
104	12666		Bari	Molfetta	6,53E+03	Package A	Omicron BA.4/5 ^a	
105	12669		Barletta-Andria-Trani	Andria	7,67E+03	Package A	Omicron BA.4/5 ^a	
106	12670		Barletta-Andria-Trani	Barletta	5,23E+03			
107	12671		Barletta-Andria-Trani	Bisceglie	7,61E+03			
108	12672		Barletta-Andria-Trani	Trani	6,03E+03	Package A	Omicron BA.4/5 ^a	
109	12698	Bari	Bitonto	2,52E+03	Package A	Omicron BA.4/5 ^a		
110	12677	Sicilia	Trapani	Trapani	1,77E+04			Package A Omicron BA.4/5
111	12678		Trapani	Mazara del Vallo	8,15E+03			
112	12679		Trapani	Marsala	5,37E+04	Package A	Omicron BA.4/5 ^a	
113	12708		Ragusa	Modica	3,60E+03			
114	12709		Ragusa	Vittoria	2,50E+04			
115	12710		Ragusa	Ragusa	2,30E+03	ME ^c		

116	12711		Caltanissetta	Gela Macchitella	5,28E+03		
117	12712		Messina	Mili Marina	4,23E+04		
118	12776		Catania	Pantano d'Arci	3,71E+04		
119	12778		Catania	Giarre	3,88E+04		
120	12779		Siracusa	Siracusa	1,26E+05		
124	12948		Agrigento	Agrigento	1,73E+04		
125	12949		Enna	Enna	5,95E+04		
126	12950		Palermo	Bagheria	1,95E+04		
127	12951		Palermo	Acqua dei Corsari	2,16E+04		
128	12952		Palermo	Fondo Verde	4,23E+04		
129	12953		Caltanissetta	Caltanissetta e San Cataldo	6,45E+04		
130	12783		Pisa	Pisa Nord - S. Jacopo	< LOD	Package A	Omicron BA.4/5
131	12784		Firenze	Empoli Pagnana	5,12E+03		
132	12785		Massa	Lavello 2	< LOD		
133	12786		Lucca	Viareggio	< LOD		
134	12787		Massa	Lavello 1	< LOD		
135	12788		Lucca	Pontetetto	8,72E+04		
136	12789		Livorno	Rivellino	1,45E+05		
137	12790	Toscana	Livorno	Rivellino	8,58E+04	Package A	Omicron BA.4/5 ^e
138	12795		Firenze	San Colombano	1,51E+03		
139	12796		Firenze	San Colombano	5,25E+03		
140	12797		Prato	Baciacavallo	1,32E+03		
141	12798		Prato	Baciacavallo	2,70E+03		
142	12799		Arezzo	Casolino - San Leo	4,53E+03		
143	12800		Grosseto	San Giovanni - Pianetto	1,13E+04		
144	12801		Pistoia	Centrale Pistoia	5,43E+03		
145	12802		Siena	Ponte a Tressa	1,57E+04		
146	12619	Umbria	Perugia	Perugia - Pian della Genna	8,13E+04		
147	12706		Perugia	Foligno Casone	2,25E+04		

148	12707		Terni	Terni	1,09E+05		
149	12769	Valle	Aosta	La Salle	3,31E+04		
150	12770	D'Aosta	Aosta	Brissogne	4,10E+04		
151	12645		Padova	Padova Ca' Nordio - centro storico	1,17E+05	Package A	Omicron BA.4/5
152	12646		Padova	Padova Ca' Nordio - zip	2,45E+05		
153	12647		Padova	Padova Guizza	3,51E+05		
154	12648		Padova	Abano Terme	1,87E+05		
155	12663	Veneto	Treviso	Treviso	2,95E+03		
156	12664		Vicenza	Vicenza Casale	3,08E+03	Package A	Omicron BA.4/5 ^a
157	12665		Venezia	Venezia Fusina	1,63E+03	Package A	Omicron BA.4/5 ^a
158	12742		Verona	Verona_collettore 1M	3,70E+04		
159	12743		Verona	Verona_collettore 3M	3,41E+04	Package A	Omicron BA.4/5 ^a
160	12744		Verona	Verona_collettore 8M	1,57E+04		

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

^b Amplification of the M gene was unsuccessful, therefore it was not possible to discriminate between BA.4 and BA.5

^c M.E. mixed electropherograms

^d the presence of a double A/G peak in correspondence to the mutation site (corresponding to presence/absence of mutation D3N in the M gene) is suggestive of the simultaneous presence of BA.4 and BA.5

^e the absence of mutation D3N in the M gene is suggestive of the presence of BA.4

- **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.2, long fragment from PCR ID_980) = G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, S477N, T478K, E484A, Q493R, Q498R, N501Y, Y505H)
- **Package C** (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

ID SAMPLES	DEL69/70	G142D	V213G	G339D	R346T	S371F	S373P	S375F	T376A	D405N	R408S	K417N	N440K	L452R	S477N	T478K	F484A	F486V	Q493R	Q498R	N501Y	Y505H	VARIANTS
8- 21-32-78- 83-84-94-98-99-100-101-102-103-104-105-108-109-112-137-156-157-159-162-163	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	Package A* (Omicron BA.4/5)
89	□	■	■	■	□	■	■	■	■	■	■	■	■	□	■	■	■	□	■	■	■	■	Package B (Omicron BA.2)
29	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	Package C (Omicron BA.4.6)

* Upon testing for the assay targeting the M gene, 23 of the 26 BA.4/BA.5 sequences were assigned to Omicron BA.5, two to BA.4, and one showed the presence of both sublineages.

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 twelfth 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 prevalence of the Omicron variant, sublineage BA.5.

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