



**Flash survey on SARS-CoV-2 variants in urban wastewater in Italy
25th Report
(Study period: September 4th to September 8th, 2023)**

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Main findings:

- During the week of 4 September to 9 September 2023, a total of 107 wastewater samples were collected from 15 Regions and 2 Autonomous Provinces (A.P.).
- Mutations characteristic of the Omicron variant were identified in 9 of these Regions/AP, while no sequencing data were obtained from the remaining areas.
- Analysis of the sequences obtained by Sanger sequencing showed that 54% of the positive samples had amino acid substitutions belonging to the Omicron XBB.1.5*/XBB.1.9* lineages, 23% to the Omicron XBB.2.3* lineage, and a final 23% to the Omicron XBB.1.16* lineage.

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. Responding to this recommendation, the Istituto Superiore di Sanità (ISS) initiated a series of "flash surveys". These surveys involve monthly sampling campaigns conducted at various locations across Italy over a short period of time. 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 summarize the findings from the 25th national Flash Survey on SARS-CoV-2 variants in wastewater samples in Italy, which was conducted from September 4th to September 8th, 2023.

Methodology

During the period from 4 September to 9 September 2023, the 25th national Flash Survey on SARS-CoV-2 variants in wastewater samples was conducted in Italy. The survey involved the collection of 107 sewage samples from 104 wastewater treatment plants (WTPs) located across 15 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 website¹. 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. The purified RNAs extracted from the samples were then sent to ISS for variant detection.

For sequencing purposes, we have designed 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, Sanger sequencing was performed on individual samples.

For variant classification we adopted a lineage classification based on 'outbreak.info'³ 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

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 \(iss.it\)](https://www.iss.it/8e5e2edb-bae0-f1b0-ee6e-08255c76484f)

² DOI [10.5281/zenodo.5758724](https://doi.org/10.5281/zenodo.5758724).

³ <https://outbreak.info/situation-reports>, date: 17/11/2023

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

Out of the 107 samples analysed, a total of 92 (86.0%) 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 $3.35E + 00$ to $8.36E + 06$ genome copies (g.c.) per liter of sewage.

Sanger Sequencing

Table 1 provides a summary of the results obtained from the long-nested PCR assay and sequencing methods. A total of 14 samples (13.1%) from 9 Regions/AP were successfully amplified using the long-nested PCR assay described above. Among these samples, high-quality sequences were obtained from 13 of them through Sanger sequencing, and one sequence was unsuccessful due to high signal noise resulting in partial sequence obtainment. Sanger sequencing confirmed that all the obtained sequences corresponded to the Omicron variant.

Analysis of wastewater samples revealed the presence of three SARS-CoV-2 lineages, as shown in Tables 1 and 2. Among these, the Omicron XBB.1.5*/XBB 1.9* lineages (which are not distinguishable in the sequenced region) were the most common, detected in 54% (7 samples) of the positive samples. Omicron XBB.2.3* and XBB.1.16* were each identified in 23% of the samples (3 samples).

For ease of understanding, the mutations have been grouped into panels or "mutation packages" as follow:

- **Package A (assigned to the lineage Omicron XBB.1.5*/XBB.1.9*)** = V83A; G142D; DEL144; H146Q/H146K; Q183E; V213E; G252V; G339H; R346T; L368I; S371F; S373P; S375F; T376A; D405N; R408S; K417N; N440K; V445P; G446S; N460K
- **Package B (assigned to the lineage Omicron XBB.1.5*/XBB.1.9*)** = Q52H; V83A; G142D; DEL144; H146Q; Q183E; V213E; G252V; G339H; R346T; L368I; S371F; S373P; S375F; T376A; D405N; R408S; K417N; N440K; V445P; G446S; F456L; N460K
- **Package C (assigned to the lineage Omicron XBB.2.3*)** = V83A, G142D, DEL144, H146Q, Q183E, V213E, D253G, G339H, R346T, L368I, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, V445P, G446S, N460K
- **Package D (assigned to the lineage Omicron XBB.1.16*)** = V83A; G142D; DEL144; H146Q; E180V; Q183E; V213E; G252V; G339H; R346T; L368I; S371F; S373P; S375F; T376A; D405N; R408S; K417N; N440K; V445P; G446S; F456L; N460K

The lineages Omicron XBB.1.5*/XBB.1.9* were detected in 7 Regions/A.P. (Emilia-Romagna, Liguria, Molise, Puglia, Sicilia, Veneto and A.P. Bolzano). Key mutations of lineage Omicron XBB.2.3* were found in 3 Regions/A.P. (Liguria, Sicilia, and Valle d'Aosta). Additionally, two Regions (Sicilia and Veneto) were found to have key mutations of Omicron XBB.1.16*.

Table 1. PCR and sequencing results

| | 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) |
|-----|-----------|----------------|-----------------------|------------------------|------------------|---|---|
| 1 | 22284 | Abruzzo | Pescara | Via Raiale | 7,42E+02 | | |
| 2 | 22285 | | Pescara | Villa Carmine | < LOD | | |
| 3 | 22286 | | Chieti | S. Martino | < LOD | | |
| 4 | 22287 | | L'Aquila | Pile | 9,20E+02 | | |
| 5 | 22288 | | Teramo | Villa Pavone | 3,24E+02 | | |
| 6 | 22391 | Basilicata | Potenza | Tiera di Vaglio | 1,18E+04 | | |
| 7 | 22392 | | Matera | Pantano | < LOD | | |
| 8 | 22350 | Emilia-Romagna | Ferrara | Ferrara - Linea 1 | < LOD | | |
| 9 | 22351 | | Ferrara | Ferrara - Linea 2 | < LOD | | |
| 10 | 22352 | | Modena | Carpi | 7,65E+03 | | |
| 99 | 22422 | | Modena | Naviglio | 8,36E+06 | | |
| 100 | 22423 | | Bologna | IDAR | 2,59E+06 | | |
| 101 | 22424 | | Bologna | Imola | 2,73E+06 | | |
| 104 | 22441 | | Forli-Cesena | Forli | 3,53E+04 | Package A + F456L | Omicron XBB.1.5*/XBB.1.9* |
| 105 | 22442 | | Cesena | Forli-Cesena | 2,17E+06 | | |
| 106 | 22443 | | Rimini - Forli-Cesena | S. Giustina | 2,26E+06 | | |
| 108 | 22567 | | Ravenna | Ravenna - Forli-Cesena | 1,59E+05 | | |
| 92 | 22383 | | Piacenza | Borgoforte | 3,15E+03 | | |
| 93 | 22384 | | Parma | Parma ovest | 1,64E+04 | | |
| 94 | 22385 | | Reggio Emilia | Mancasale | 2,78E+04 | | |
| 102 | 22451 | | Friuli-Venezia Giulia | Pordenone | Cordenons | 4,28E+06 | |
| 103 | 22452 | Udine | | Udine | 1,75E+06 | | |
| 107 | 22453 | Trieste | | Servola | 1,93E+06 | | |
| 11 | 22301 | Lazio | Latina | Latina Loc Latina Est | 2,10E+04 | | |
| 12 | 22302 | | Roma | Anzio - Colle Cocchino | 2,01E+04 | | |

| | | | | | | | |
|----|-------|-----------|--|-------------------------------|----------|--|---------------------------|
| 13 | 22303 | | Latina | Aprilia (Via del Campo) | 1,19E+03 | | |
| 14 | 22304 | | Roma | Guidonia - Ponte Lucano | 7,80E+02 | | |
| 15 | 22305 | | Roma | Pomezia - Via Cincinnato | 1,48E+03 | | |
| 16 | 22306 | | Roma | Velletri (LA CHIUSA-SORBO) | 1,33E+03 | | |
| 17 | 22307 | | Viterbo | Viterbo - Strada Bagni | 6,65E+03 | | |
| 18 | 22386 | | Roma | Civitavecchia Fiumaretta | < LOD | | |
| 19 | 22312 | | Savona | Savona | 9,20E+03 | | |
| 20 | 22313 | | Genova | Voltri | 3,71E+04 | | |
| 21 | 22314 | | Genova | Quinto | 3,99E+04 | | |
| 22 | 22315 | | Genova | Rapallo | 3,50E+04 | | |
| 23 | 22316 | | Genova | Sestri P | 6,60E+04 | | |
| 24 | 22317 | | Genova | Sturla | 6,76E+04 | | |
| 25 | 22318 | | Genova | Darsena | 5,18E+04 | | |
| 26 | 22319 | Liguria | Genova | Punta Vagno Genova | 4,93E+04 | | |
| 27 | 22320 | | Genova | Valpolcevera | 3,21E+03 | | |
| 28 | 22321 | | Imperia | Sanremo - località Capo Verde | 1,52E+04 | | |
| 29 | 22322 | | La Spezia | La Spezia | 1,89E+03 | | |
| 30 | 22323 | | Genova | Pegli | 2,07E+03 | Package B + T3071 + L445F ^a | Omicron XBB.1.5*/XBB.1.9* |
| 31 | 22324 | | Savona | Borghetto Santo Spirito | 1,19E+04 | | |
| 95 | 22457 | | Genova | Recco | 2,65E+04 | Package C + K182N + K444R | Omicron XBB.2.3* |
| 32 | 22278 | | Milano | Milano Nosedo | < LOD | | |
| 33 | 22279 | | Milano | Milano San Rocco | < LOD | | |
| 34 | 22280 | | Como | Como | < LOD | | |
| 35 | 22281 | | Pavia | Pavia | < LOD | | |
| 36 | 22282 | | Como - Lecco - Milano - Monza e della Brianza | Monza | < LOD | | |
| 37 | 22283 | Lombardia | Pavia | Vigevano | < LOD | | |
| 38 | 22337 | | Bergamo | Bergamo | < LOD | | |
| 39 | 22339 | | Brescia | Verziano | 1,33E+03 | | |
| 40 | 22341 | | Cremona | Citta di Cremona | 1,18E+03 | | |
| 88 | 22697 | | Sondrio | Sondrio | 5,98E+03 | | |

| | | | | | | | |
|----|-------|--------------|---------------|----------------------------------|----------|---------------------------|---------------------------|
| 41 | 22334 | Marche | Pesaro-Urbino | Borgheria | 1,94E+04 | | |
| 42 | 22335 | | Pesaro-Urbino | Ponte Metauro | 1,14E+04 | | |
| 43 | 22336 | | Ancona | Zipa | 2,40E+04 | | |
| 44 | 22338 | | Ancona | Falconara | 7,79E+03 | | |
| 45 | 22289 | Molise | Campobasso | Campobasso - San Pietro | 3,35E+00 | | |
| 46 | 22290 | | Campobasso | Termoli - località Porto | < LOD | Package A + A344T+ R403K | Omicron XBB.1.5*/XBB.1.9* |
| 47 | 22291 | | Campobasso | Termoli - località Pantano Basso | < LOD | | |
| 48 | 22265 | A.P. Trento | Trento | Trento nord | 1,50E+05 | | |
| 49 | 22266 | | Trento | Trento sud | 9,89E+04 | | |
| 50 | 22267 | | Trento | Rovereto | 1,60E+05 | | |
| 89 | 22437 | A.P. Bolzano | Bolzano | IDA Bolzano | 1,11E+04 | | |
| 90 | 22438 | | Bolzano | IDA Merano | 1,18E+04 | Package A + L455F + F456L | Omicron XBB.1.5*/XBB.1.9* |
| 91 | 22447 | | Bolzano | IDA Termeno | 6,80E+04 | | |
| 51 | 22249 | Piemonte | Torino | Castiglione Torinese | 7,08E+03 | | |
| 52 | 22250 | | Biella | Biella Nord | 5,68E+02 | | |
| 53 | 22251 | | Biella | Biella Sud | 1,11E+03 | | |
| 54 | 22252 | | Novara | Novara | 1,29E+03 | | |
| 55 | 22275 | | Alessandria | Alessandria | 1,57E+04 | | |
| 56 | 22276 | | Asti | Asti | 2,63E+03 | | |
| 57 | 22277 | | Cuneo | Cuneo | 5,45E+03 | | |
| 58 | 22258 | Puglia | Bari | Bari Est | 1,73E+03 | | |
| 59 | 22259 | | Bari | Bari Ovest | 2,37E+03 | Package B ^a | Omicron XBB.1.5*/XBB.1.9* |
| 60 | 22260 | | Taranto | Taranto Bellavista | 7,92E+02 | | |
| 61 | 22261 | | Taranto | Taranto Gennarini | 8,83E+02 | | |
| 62 | 22262 | Sicilia | Trapani | Trapani | 6,56E+03 | | |
| 63 | 22263 | | Trapani | Mazara del Vallo | 1,26E+04 | | |
| 64 | 22264 | | Trapani | Marsala | 1,83E+04 | | |
| 65 | 22330 | | Ragusa | Modica | 3,75E+02 | | |
| 66 | 22332 | | Ragusa | Ragusa | 4,33E+03 | | |
| 67 | 22333 | | Caltanissetta | Gela Macchitella | 4,38E+03 | | |

| | | | | | | | |
|----|-------|---------------|---------------|------------------------------------|----------|--|---------------------------|
| 68 | 22354 | | Agrigento | Agrigento | 3,93E+04 | | |
| 69 | 22355 | | Enna | Enna | 5,01E+04 | | |
| 70 | 22356 | | Palermo | Bagheria | 2,13E+04 | Package B ^a | Omicron XBB.1.5*/XBB.1.9* |
| 71 | 22357 | | Palermo | Acqua dei Corsari | 2,07E+04 | | |
| 72 | 22358 | | Palermo | Fondo Verde | 4,11E+04 | Package D | Omicron XBB.1.16* |
| 73 | 22359 | | Caltanissetta | Caltanissetta e San Cataldo | 2,26E+04 | Package C | Omicron XBB.2.3* |
| 96 | 22399 | | Catania | Pantano d'Archi | 1,45E+04 | | |
| 97 | 22400 | | Catania | Giarre | 1,49E+04 | | |
| 98 | 22401 | | Siracusa | Siracusa | 1,00E+04 | | |
| 74 | 22300 | Umbria | Perugia | Perugia - Pian della Genna | 1,99E+04 | M.E. (partial sequence, from R346T to N460K) ^b | Not assigned |
| 75 | 22325 | Valle d'Aosta | Aosta | La Salle | 6,24E+02 | | |
| 76 | 22326 | | Aosta | Brissogne | 4,40E+02 | Package C + V70A + G184V | Omicron XBB.2.3* |
| 78 | 22254 | Veneto | Padova | Padova Ca' Nordio - centro storico | 3,63E+04 | | |
| 79 | 22255 | | Padova | Padova Ca' Nordio - zip | 4,86E+04 | | |
| 80 | 22256 | | Padova | Padova Guizza | 3,70E+04 | | |
| 81 | 22257 | | Padova | Abano Terme | 5,10E+04 | | |
| 82 | 22271 | | Vicenza | Vicenza Casale | 1,35E+04 | Package B ^a | Omicron XBB.1.5*/XBB.1.9* |
| 83 | 22272 | | Treviso | Treviso | 1,14E+04 | Package D + L452Q | Omicron XBB.1.16* |
| 84 | 22273 | | Venezia | Venezia Fusina | 7,84E+03 | | |
| 85 | 22309 | | Verona | Verona_collettore 1M | 1,32E+04 | Package D | Omicron XBB.1.16* |
| 86 | 22310 | | Verona | Verona_collettore 3M | 1,09E+04 | | |
| 87 | 22311 | | Verona | Verona_collettore 8M | 1,76E+04 | | |

^a Package B may suggest the presence of the Omicron EG.5 sublineage. This last sublineage is characterised by the presence of the 'Q52H' mutation in approximately 92.7% and the 'F456L' mutation in about 96.1% of its sequences. The Omicron XBB.1.5* lineage and the Omicron XBB.1.9* lineage may also carry the 'Q52H' mutation at rates of 0.2% and 71.3% respectively, and the 'F456L' mutation at rates of 7.1% and 76.8% respectively (<https://outbreak.info/situation-reports>, date: 17/11/2023).

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

Table 2. Sanger sequencing results

| ID SAMPLES | Q52H | V83A | G142D | DEL 144 | H146Q/H146K | E180V | Q183E | V213E | G252V | D253G | G339H | R346T | L368I | S371F | S373P | S375F | T376A | D405N | R408S | K417N | N440K | V445P | G446S | F456L | N460K | VARIANTS |
|----------------|------|------|-------|---------|-------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------------------------------------|
| 46, 90, 104 | | | | | | | | | | | | | | | | | | | | | | | | | | Package A (Omicron XBB.1.5*/XBB.1.9*) |
| 30, 59, 70, 82 | | | | | | | | | | | | | | | | | | | | | | | | | | Package B (Omicron XBB.1.5*/XBB.1.9*) |
| 73, 76, 95 | | | | | | | | | | | | | | | | | | | | | | | | | | Package C (Omicron XBB.2.3*) |
| 72, 83, 85 | | | | | | | | | | | | | | | | | | | | | | | | | | Package D (Omicron XBB.1.16*) |

Limitations of the study

The geographical and population coverage of this flash survey was not comprehensive, as it encompassed 17 out of 21 of the Italian Regions/Autonomous Provinces.

It is important to note that the molecular analytical methods used for complex environmental matrices, such as wastewater, can face challenges due to factors such as low viral concentrations, inadequate analyte recovery, and/or PCR amplification inhibition. Consequently, both the detection/quantification and PCR amplification for sequencing may yield false negatives, making it challenging to achieve molecular characterization and variant detection for all samples.

Partial sequencing of 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 assignment 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 results of this survey indicate that the Omicron variant is the sole SARS-CoV-2 variant in Italy, with the Omicron XBB.1.5*/XBB.1.9* lineages being the most prevalent. The Omicron XBB.2.3* and XBB.1.16* lineages have also been detected. 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);
- **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, Franca Palumbo, Gianluca Borlone, Marco Guercio (IREN);
- **Friuli Venezia Giulia:** Marika Mariuz, Gabriella Trani (Direzione Centrale Salute FVG); Anna Pariani (LABORATORIO HERATech di Sasso Marconi –BO);
- **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, Franca Palumbo, 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:** Giuseppe Di Vittorio, Onofrio Mongelli (Regione Puglia); Osvolda De Giglio, Francesca Apollonio, Francesco Triggiano, Maria Teresa Montagna (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 (Laboratorio di Igiene e Virologia Ambientale Dipartimento di Biologia Università di Pisa); Matteo Ramazzotti (Dipartimento di Scienze Biomediche Sperimentali e Cliniche, Università degli Studi di Firenze), Alberto Antonelli (SOD microbiologia e virologia, azienda ospedaliera universitaria Careggi, Firenze);
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