

Epidemiological characteristics of non-polio enteroviruses in respiratory infections: An Italian multicentre retrospective study, 2022–2023

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1. Introduction

Laboratory-based activities are regarded as a fundamental component of infectious disease trend monitoring and rely on data generated in clinical and public health laboratories (Bean and Martin, 2001). The architecture of a synergistic laboratory system to detect and control pathogens has the potential to be applied to pathogens not yet under investigation, as exemplified by the case of non-polio enteroviruses

(NPEV) (Fischer et al., 2022). These single-stranded RNA viruses are highly contagious and are transmitted mainly through the fecal-oral and airborne route, being associated with several human diseases (Bubba et al., 2020). Historically, enteroviruses and rhinoviruses were classified as separate genera, but due to their closely related genome organisation and structure, they have been merged into a single genus named *Enterovirus*. Indeed, the high genetic similarity between enteroviruses and rhinoviruses in particular in the 5' non-translating region (5' NTR)

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remains a pitfall for molecular diagnostic tools. In fact, a good diagnostic method for routine laboratory confirmation of respiratory viruses should be able to correctly distinguish enterovirus from rhinovirus and detect the large number of different enterovirus types for accurate diagnosis, but this is not always achieved (Andrés et al., 2019).

Despite the high burden of NPEV infections and their associated diseases, which are particularly severe in the youngest children, no ad hoc NPEV surveillance has been established by international health organisations to date. Currently, epidemiological and molecular data on NPEV are emerging from single studies, which, however, are not comprehensive in their setting and case definition, leaving gaps in data on NPEV burden of disease (CDC, 2005; Bingjun et al., 2008; Molet et al., 2016; Benschop et al., 2016; Andrés et al., 2019; Piralla et al., 2020; Enterovirus Study, collaborators, 2020; Fischer et al., 2022). More than one hundred NPEV genotypes have been identified as being able to cause several clinical syndromes, ranging from mild to severe illness. Recently, there has been an increase in the number of cases of enterovirus D68 (EV-D68), which has been associated with outbreaks of severe respiratory infections (Benschop et al., 2021; Fall et al., 2022; Midgley et al., 2015; Shah et al., 2021) and poliomyelitis-like acute flaccid myelitis (Carrion Martin et al., 2017; Eunson, 2017; Pellegrinelli et al., 2021; Hodcroft et al., 2022). In addition, coxsackievirus (CV) B3-B5 have been associated with outbreaks of myocarditis (Singanayagam et al., 2023), and echovirus 11 (E-11) has been linked to fatal neonatal infections (Zheng et al., 2013; Piralla et al., 2023; Hu et al., 2023; Grapin et al., 2023). This highlights the necessity to narrow the gap in our comprehension of NPEV epidemiology, with a specific emphasis on viral tracking and molecular characteristics.

In Italy, poliovirus surveillance has been established since 1997 through the monitoring of acute flaccid paralysis and environmental surveillance of wastewater samples (Pellegrinelli et al., 2017), as part of the Global Polio Eradication Initiative (<https://polioeradication.org/>). However, there is currently no surveillance of NPEV in the clinical setting. Several frameworks for NPEV surveillance have been developed in other countries, such as the TYPENED (Niesters et al., 2013) and ICARES (Groeneveld et al., 2017) data sharing systems. In these contexts, scientists should be able to determine the distribution and circulation of NPEV in communities and hospitalised patients by analysing virological data extracted from virological databases of clinical and public health laboratories that routinely perform molecular testing for NPEV. The routine collection and analysis of a minimum set of data, including age group, sex, hospitalisation ward, date and type of sample collected for analysis, using a centralised real-time database system, may allow the identification of spatial, temporal and demographic changes in NPEV prevalence and the early detection of any NPEV outbreak. This approach has recently been demonstrated in Italy (Pellegrinelli et al., 2024) and elsewhere (CDC, 2005; Benschop et al., 2016). Although the respiratory tract has not traditionally been considered a primary site of infection for NPEV, recent studies (CDC, 2005; Benschop et al., 2016) have highlighted the role of these viruses in respiratory disease. In the context of the Working Group on Respiratory Virus Infections (GLiViRe) of the Italian Association of Clinical Microbiologists (AMCLI), a multicentre retrospective observational study was conducted to describe the spatial, temporal and genotypic distribution of NPEV in Italy over a one-year period (from July 2022 to June 2023), in order to study the epidemiology of NPEVs specifically in the context of respiratory infections, which are still understudied.

2. Materials and methods

2.1. Data collection

In June 2023, an email invitation was extended to all microbiological laboratories belonging to the GLiViRe working group, requesting their participation in a study on NPEV-positive respiratory samples. For each laboratory that agreed to participate in the study, information was

requested on the molecular methods employed for the detection and characterisation of NPEV (listed in Table 1). Subsequently, laboratories were asked to extract data from their real-life diagnostic activities on the molecular detection of NPEV. As the molecular assays used by laboratories were able to distinguish between the NPEV genome and that of rhinoviruses, this study analysed virological and epidemiological data only from samples that tested positive for NPEV. Data about NPEV were retrieved from diagnostic databases and analysed retrospectively. The queries used to extract the data from the databases were as follows: (a) requests for NPEV-RNA detection in respiratory specimens (nasopharyngeal swabs, aspirates, bronchoalveolar lavages) collected from patients presenting with symptoms of influenza-like illness (ILI) or acute respiratory infection (ARI) with no age restriction; and b) requests submitted between July 4, 2022 (week 2022–27) and June 26, 2023 (week 2023–26).

Data on the number of NPEV-RNA positive samples and the total number of respiratory samples tested for NPEV by week were collected for each laboratory. Where available, data on NPEV genotypes were also collected. For NPEV-RNA positive samples, information on the age and sex of the patient was also collected. The study was conducted in accordance with the Declaration of Helsinki (1975), rev. 2000 and the data were handled anonymously.

2.2. Data analysis

The number of NPEV detections by week were reported. The NPEV detection rate per macro-geographic area and age group were expressed as a crude proportion, with the corresponding 95% confidence interval (95% CI) calculated by the Mid-P exact test, assuming a normal distribution. Data from laboratories that did not distinguish enterovirus detection from rhinovirus were excluded from the calculation of the NPEV positivity rate.

The interquartile range (IQR) was calculated as the difference between the first and third quartiles of the distribution of the variables under study. The statistical significance of the differences between the proportions in the various groups was determined using the Mid-P exact test based on the binomial distribution. For continuous variables, the paired *t*-test was employed. The statistical analysis was conducted using the Open Source Epidemiologic Statistics for Public Health OpenEpi, version 3.03 (Dean et al., 2006). The following definitions were established: 1) The peak value of NPEV is defined as the maximum number of NPEV-positive samples observed in a given week during the epidemic time-series; 2) The peak time is defined as the week when the peak value is attained; 3) The onset of the NPEV epidemic is defined as the time at which the fraction of NPEV-positive samples exceeds a specific threshold value; 4) The intensity duration of the NPEV epidemic is defined as the number of weeks where the number of NPEV-positive samples exceeds a specific threshold. The threshold value was defined as the average number of NPEV-positive samples observed during the study period, plus two standard deviations, a proposal that was first made by the CDC (CDC, 2023) and described also by Tabataba et al. (2017). A *p*-value of less than 0.05 was considered to be statistically significant (two-tailed test).

3. Results

A total of 12 laboratories participated in the study. Of these laboratories, 10 (namely lab. No. 1–7, 10–12) were able to distinguish between NPEV and rhinovirus, whereas two laboratories (namely lab. No. 8 and 9) were only able to perform assays that targeted NPEV, which was indistinguishable from rhinovirus (Table 1). The molecular characterisation of NPEV by sequencing analysis was conducted on a routine basis in two of the 12 laboratories (lab. No. 1 and 2). Four of the 12 laboratories (lab No. 3, 4, 7 and 8) conducted sequencing analysis on selected NPEV-positive cases. In total, the laboratories that participated in the study were located in nine of the 20 Italian regions. The laboratories

Table 1

Affiliations and location of the laboratories of the GLiViRe working group and methods used for molecular detection and characterization of NPEV by the laboratories.

No.	Laboratory's affiliation	Region (macro-area) ^a	NPEV-RNA detection method	NPEV typing method
1	Department of Biomedical Sciences for Health, University of Milan, Milan, Italy	Lombardy (North-West)	Home-made (Asner et al., 2014)	Routine typing (Nix et al., 2006)
2	Microbiology and Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy	Lombardy (North-West)	Home-made (Piralla et al., 2020)	Routine typing (Nix et al., 2006; Giardina et al., 2022)
3	Microbiology and Virology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy	Lombardy (North-West)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	Typing of selected cases (Nix et al., 2006)
4	Laboratory of Medical Microbiology and Virology University Hospital, ASST Sette Laghi, Varese, Italy	Lombardy (North-West)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene) and Biofire FilmArray (BioMerieux) ^b	Typing of selected cases (Nix et al., 2006; Giardina et al., 2022)
5	Laboratory of Microbiology and Virology, Provincial Hospital of Bolzano (SABES-ASDAA), Bolzano, Italy	Trentino Alto-Adige (North-East)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	No
6	UOC Microbiology Treviso Hospital, Department of specialist and laboratory medicine, AULSS 2 La Marca, Italy	Veneto (North-East)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	No
7	Microbiology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy	Emilia Romagna (North-East)	Enterovirus Elite Mgb Kit - Elitech Group Real Time Pcr and Filmarray (BioMerieux)	Typing of selected cases (Nix et al., 2006)
8	Virology Unit, Azienda Ospedaliero Universitaria delle Marche, Ancona, Italy	Marche (Centre)	FTD Respiratory Pathogens 21 Assay (Siemens-Healthineers) ^b	Typing of selected cases (Nix et al., 2006; Giardina et al., 2022)
9	Laboratory of Virology, National Institute for Infectious Diseases "Lazzaro Spallanzani" IRCCS, Rome, Italy	Lazio (Centre)	QIAstat-Dx Respiratory SARS-CoV-2 Panel (Qiagen) ^b	No
10	Virology and Mycobacteria UOS, Microbiology and Diagnostic Immunology UOC, Bambino Gesù Children Hospital IRCCS, Roma, Italy	Lazio (Centre)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	No
11	UOC Microbiology and Virology, Cotugno Hospital AORN dei Colli, Naples, Italy	Campania (South)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	No
12	Virology Laboratory - Microbiology and Virology Unit - University of Bari - Policlinic of Bari, Bari, Italy	Apulia (South)	Allplex™ Respiratory Panel Assays on All-in-One Platform (Seegene)	No

^a According to the NUTS classification, <https://ec.europa.eu/eurostat/documents/3859598/15193590/KS-GQ-22-010-EN-N.pdf>.^b This method does not allow to discriminate between rhinovirus and enterovirus.

were distributed across four macro-areas of Italy: the north-west (Lombardy), the north-east (Trentino Alto Adige, Veneto and Emilia Romagna), the centre (Marche and Lazio) and the south (Apulia and Campania). A detailed overview of laboratory names and their location by region and macro-area is reported in Table 1.

A total of 35,532 respiratory specimens were analysed for the presence of NPEV-RNA during the study period. Of the total number of samples analysed, 33,553 were tested using molecular assays capable of detecting NPEV, while 1979 were tested using assays capable of detecting both NPEV and rhinovirus, and thus excluded from further analysis. A total of 987 respiratory samples tested positive for NPEVs, resulting in an overall positivity rate of 2.9% (95% CI: 2.8–3.1%). The NPEV positivity rate in the outpatient care setting was 8.3% (238/2852), while the NPEV positivity rate ranged from 0.2% to 5.5% in samples collected in consideration of the macro-area. The EVs positivity rate was 3.7% in north-west Italy, 0.9% in north-east Italy, 5.4% in central Italy and 0.2% in the participating centers of southern Italy. Fig. 1 shows the number of NPEV-positive samples detected by macro-area. Nearly 57% of respiratory samples that tested positive for NPEV-RNA were collected from males ($p < 0.05$). The median age of NPEV-positive patients was 3 years (IQR: 6 years). The cumulative NPEV-positivity rate observed in paediatric individuals (aged below 15 years) was 78.7% (777/987). In detail, the NPEV-positivity rate was 12.6% (124/987) and 40.7% (402/987) in children below one year of age and in those aged between one and three years, respectively. In individuals aged 65 years and above, the NPEV-positivity rate was 11.5% (114/987) (Fig. 2).

A total of 15.8% (156/987) of NPEV-positive samples were also positive for rhinovirus genome detection. As it has been documented previously (Andrés et al., 2019), a degree of cross-reactivity with rhinoviruses is to be expected, given the similarity between certain rhinoviruses and NPEV in the 5' UTR. Therefore, rhinovirus was excluded from the co-detection analysis. The presence of at least one additional respiratory virus other than rhinovirus was identified in 42.6% (421/987) of NPEV-positive samples. Adenovirus was the most frequently detected virus (133/421; 31.6%), followed by respiratory

syncytial virus (52/421; 12.3%), metapneumovirus (48/421; 11.4%), and influenza virus (43/421; 10.2%). In contrast, the pathogen most rarely detected in co-detection with NPEV was SARS-CoV-2, occurring in only 14 of the 421 NPEV-positive samples (3.3%). The percentage of co-detection was also stratified by age group, with the results showing that in individuals aged 0–4 years and in individuals aged 5–15 years, co-detections accounted for 47.1% and 33.7%, respectively. In adults aged 15–64 years and in those aged 65 years and older, the presence of additional viruses besides NPEV was observed in 19.0% and 4.3% of respiratory samples, respectively.

Molecular characterisation by sequence analysis was successfully conducted for 177 out of 987 NPEV-positive samples, representing 17.9% of the total number of identified NPEV at the national level. Conversely, 810 respiratory samples, despite testing positive for NPEV, lacked sufficient viral material for successful typing.

A total of 18 different EV types were identified. EV of group A (CV-A2, CV-A4, CV-A5, CV-A6, CV-A9, CV-A10, CV-A16) accounted for 14.1% (25/177) of the total, while EV of group B (E-3, E-11, E-18, CV-B2, CV-B3, CV-B4, CV-B5) accounted for 24.3% (43/177). Group C (CV-A21, EV-C105, EV-C109) accounted for 1.7% (3/177), and group D (EV-D68) accounted for 59.9% of EVs.

The temporal distribution of the different types of NPEV revealed changes in the predominant genotypes by week, with the emergence of a set of genotypes, particularly EV-D68 and E-11, which exhibited epidemic circulation (Fig. 3). An analysis of the distribution of NPEV by week and by geographical macro-area revealed that NPEV epidemic waves were observed, except for south Italy. Overall, at the national level, the weekly number of NPEV-positive samples during the study period ranged from 1 to 34, with a weekly average value of 12 and a standard deviation of 6.6. During the study period, the distribution of the number of NPEV-positive samples identified in the Italian laboratories participating in the study demonstrated the occurrence of three distinct NPEV epidemics, exceeding the threshold value for the weekly number of NPEV. Fig. 1 illustrates the temporal progression of the NPEV epidemic waves. The first epidemic started in week 2022–38, reached its peak in week 2022–43, and persisted for 11 weeks (Fig. 1). At the first

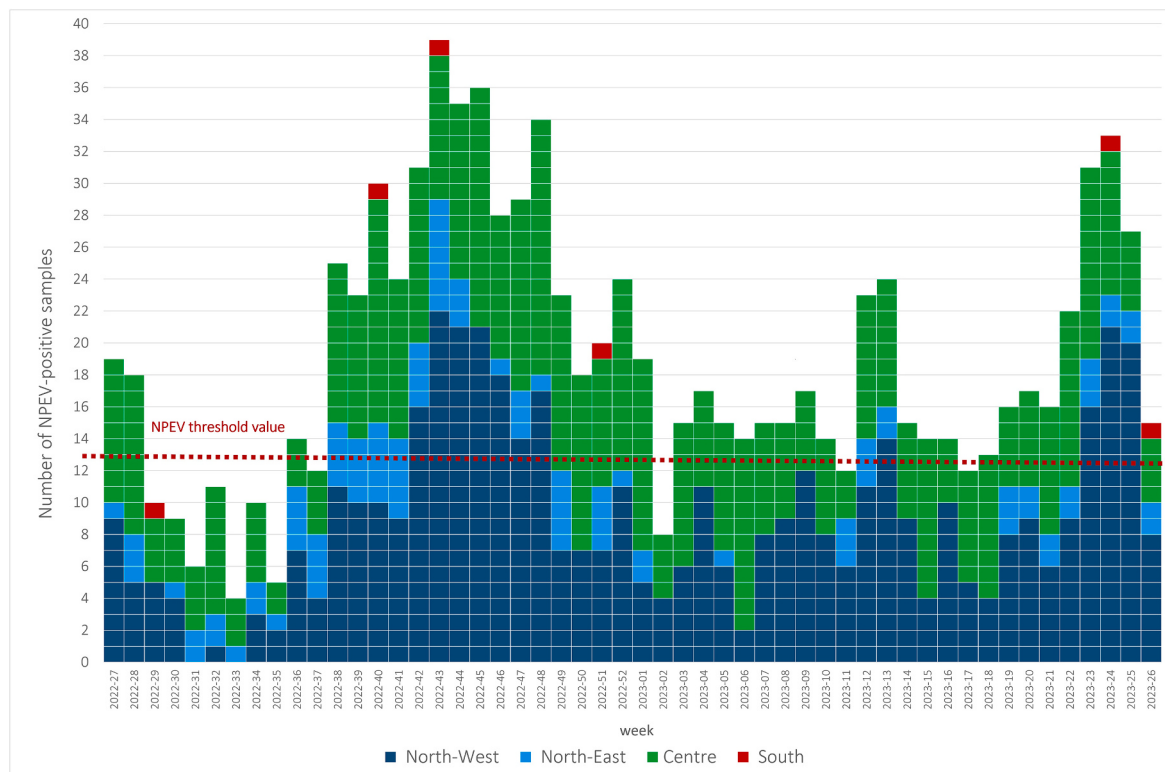


Fig. 1. Number of NPEV-positive samples detected from week 2022-27 to week 2023-26 by week and by macro-area: north-west Italy (Lombardy), north-east Italy (Trentino Alto Adige, Veneto, and Emilia Romagna), central Italy (Marche and Lazio) and south Italy (Apulia and Campania).

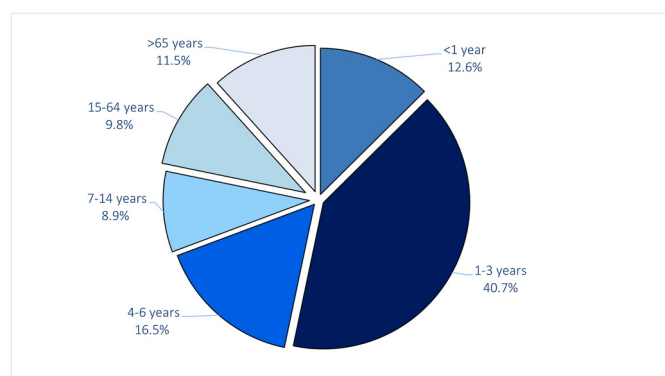


Fig. 2. Percentage of NPEV-positive respiratory samples by age group from week 2022-27 to week 2023-26 in Italy.

EV epidemic peak (week 2022–43), the overall NPEV positive rate was 9.4% (39/414, 95% CI: 6.7–12.6%), with notable variation across the macro-areas (Fig. 1). The prevalence of NPEV was 10.7% in the north-west of Italy, 4.3% in the north-east, 5.5% in the centre and 2.3% in the south. During the initial NPEV epidemic (weeks 2022-38/2022-49), EV-D68 was the most prevalent type of NPEV (Fig. 3). The second and third NPEV epidemic waves exhibited a lower intensity (length: 7 weeks) than the first epidemic (length: 11 weeks) (Fig. 1). The second NPEV wave commenced in week 2023-07 and reached its peak in week 2023–13 (Fig. 1). At this peak (week 2023–13), the overall NPEV positivity rate was 4.1% (24/58). The overall NPEV positivity rate at the macro-level was 4.5% in north-west Italy, 0.9% in north-east Italy, 5% in central Italy, and 0% in south Italy. During the second NPEV epidemic (weeks 2023-07/2023-14), multiple EV types circulated, including E–18, CV-B and E–11 (Fig. 3). The third NPEV wave showed the onset in week 2023-19 and peaked in week 2023–24 (Fig. 1): at the peak, the

overall NPEV positivity rate was 8.5% (33/386, 95% CI: 6.1–11.9%), with notable regional variation. The highest rates were observed in north-west Italy (10.5%), north-east Italy (1.6%), central Italy (6.6%) and south Italy (1.7%). During the third EV epidemic (weeks 2023-19/2023-26), E–11 was the most prevalent genotype, accounting for the majority of cases (Fig. 3).

4. Discussion

At present, NPEV surveillance has not yet been implemented in Italy, as well as in most European countries. This study was conducted by the GLiViRe working group and aimed to describe the spatial, temporal and genotype distribution of NPEV in Italy over a one-year period (from July 2022 to June 2023). A total of 12 laboratories, distributed across nine of the 20 Italian regions, participated in this multi-centre, retrospective, observational study. Data on 35,532 respiratory samples analysed for NPEV-RNA over the course of a year were collected.

The epidemiological features of 987 NPEV-RNA positive specimens were analysed in this study. As observed elsewhere (Cabrerizo et al., 2017), 12.6% of NPEV-positive samples were collected from infants below one year of age and 40.7% were from children between one and three years old. This indicates that approximately half of all NPEV infections occurred in toddlers and below, a demographic group that is particularly susceptible to severe clinical manifestations (Tseng et al., 2020; Fischer et al., 2022). At the time of testing, the clinical picture of individuals who tested positive for NPEV included respiratory infections, such as ILI and ARI. However, no clinical follow-up or information on the eventual progress or worsening of the infection was available, which represents a limitation of this study. As anticipated in the literature, at least another pathogen was identified in nearly 65% of samples that tested positive to NPEV-RNA (Meskill and O'Bryant, 2020). However, the clinical significance of these co-infections remains a topic of debate (Meskill and O'Bryant, 2020), and it was not investigated in the present study.

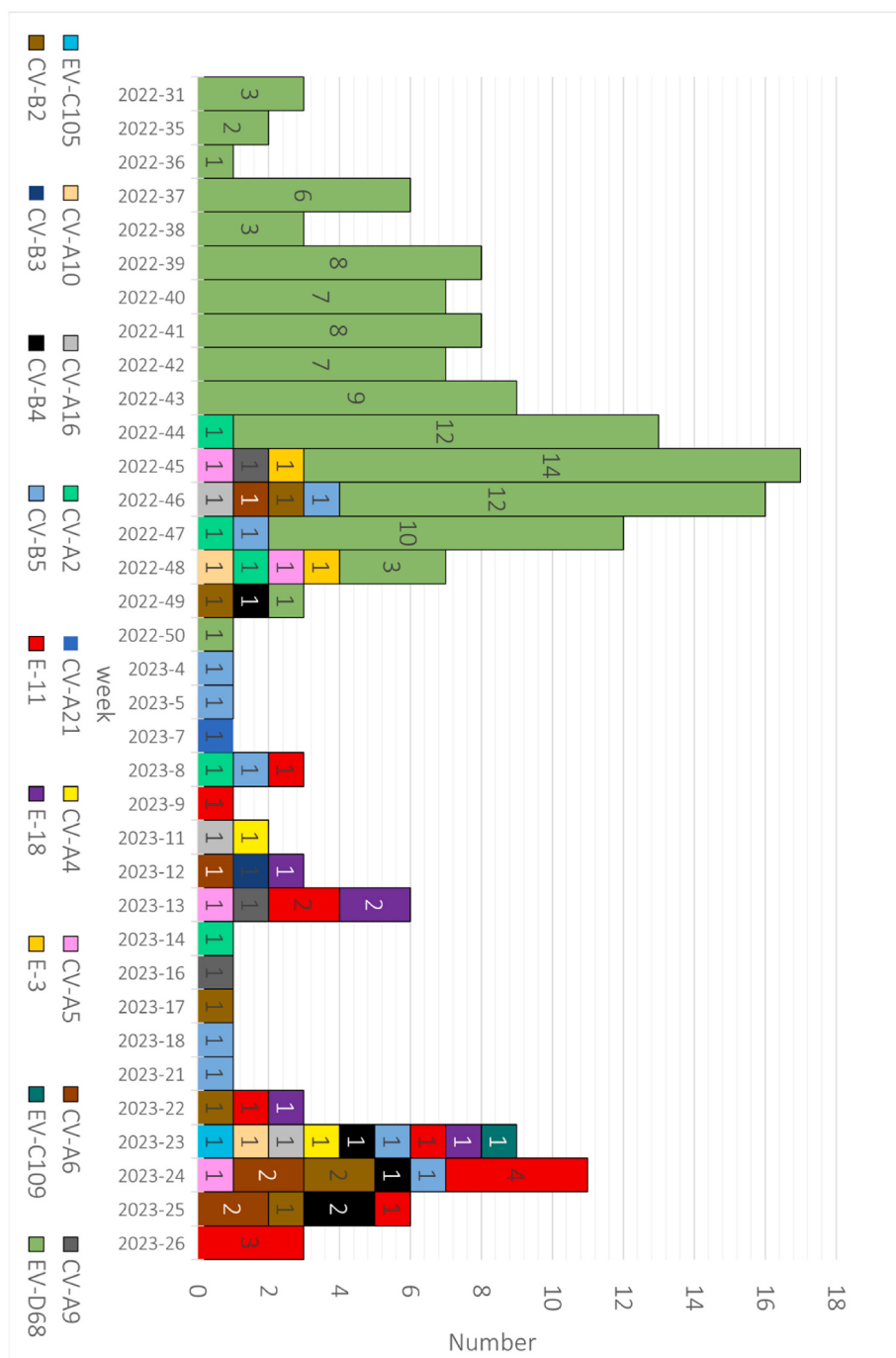


Fig. 3. Distribution of NPEV genotypes identified from week 2022-27 to week 2023-26 in Italy.

Eighteen different genotypes were identified during the study period, with at least three distinct epidemics observed. The first epidemic was caused by EV-D68 and occurred in September 2022, during an European EV-D68 outbreak reported in 18 countries and previously described by [Benschop et al. \(2021\)](#). The second epidemic peaked at the end of March 2023, revealing the co-circulation of CV-B, E-18 and E-11. The latter genotype became the most prevalent since April 2023, coinciding with the third outbreak identified here at the same time as a series of severe and fatal E-11 neonatal infections was disclosed in Europe ([Piralla et al., 2023](#); [Grapin et al., 2023](#); [WHO, 2023](#); [Piralla et al., 2024](#)).

In 42.6% of NPEV-positive samples, at least one additional respiratory virus distinct from rhinovirus was identified. The cumulative frequency of detection of adenovirus, respiratory syncytial virus,

metapneumovirus, and influenza was 65.5%, with a particularly high frequency observed in young individuals aged 0–4 years (47.1%) and those aged 5–15 years (33.7%). The extant literature does not provide sufficient evidence to support the hypothesis that the severity of clinical syndromes can be correlated with the number of respiratory viruses detected. This is due to the fact that the mechanisms underlying disease virulence in co-infections remain unclear ([Asner et al., 2014](#)). Indeed, a systematic review and meta-analysis comprising twenty-one studies and 4,280 patients revealed no significant difference in clinical disease severity between viral co-infections and single respiratory infections ([Asner et al., 2014](#)). However, an elevated mortality rate was observed in preschool children with co-infection compared to other age groups ([Asner et al., 2014](#)).

The laboratory data indicates that the routine typing of NPEV is not currently performed, which reveals the necessity to reinforce the molecular typing of NPEV in Italy. This can be achieved by implementing harmonised guidelines and protocols for the identification of outbreaks and the phylogenetic analysis of emerging variants, as well as by implementing next generation sequencing, which may shed light on the occurrence of recombinant genotypes (Lukashev et al., 2014; Muslin et al., 2019). A limitation of this study is the unavailability of clinical data, which did not allow us to assess the clinical impact of NPEV. Nevertheless, it can be considered as a pilot study to elucidate the epidemiological characteristics of NPEV, to provide a baseline assessment of their circulation and to guide future studies. Furthermore, establishing the precise role of NPEV in lung inflammation in instances of co-infection is a complex undertaking. To this end, future research is required, including the evaluation of viral load in specific tissue or compartments and the utilisation of histopathological and experimental studies.

In conclusion, this study has demonstrated that data on the epidemiology of NPEV should be obtained by collecting, collating and analysing real data from virological databases of clinical and public health laboratories. Genetic investigation of NPEV strains is crucial for the detection of emerging genotypes responsible for outbreaks in the area under study.

Finally, the integration of epidemiological data with clinical data could enable to better define clinical manifestations and outcomes associated to different NPEV types. It is therefore recommended that all these aspects will be taken into account in the design of future NPEV surveillance activities.

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CRediT authorship contribution statement

Laura Pellegrinelli: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Federica A.M. Giardina:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Federica Novazzi:** Writing – original draft, Investigation, Data curation, Conceptualization. **Elisa Vian:** Visualization, Software, Methodology, Investigation. **Valeria Biscaro:** Visualization, Software, Methodology, Investigation. **Cristina Russo:** Visualization, Software, Methodology, Investigation. **Stefania Ranno:** Visualization, Software, Methodology, Investigation. **Sara Uceda Renteria:** Visualization, Software, Methodology, Investigation. **Annapaola Callegaro:** Visualization, Software, Methodology, Investigation. **Elisabetta Pagani:** Visualization, Software, Methodology, Investigation. **Elisa Masi:** Visualization, Software, Methodology, Investigation. **Claudia Tiberio:** Visualization, Software, Methodology, Investigation. **Martina Esposito:** Visualization, Software, Methodology, Investigation. **Katia Marinelli:** Visualization, Software, Methodology, Investigation. **Stefano Menzo:** Visualization, Software, Methodology, Investigation. **Sandro Binda:** Visualization, Software, Methodology, Investigation. **Francesca Rovida:** Visualization, Software, Methodology, Investigation. **Nicasio Mancini:** Validation, Software, Methodology, Investigation. **Anna Maria Colacicco:** Visualization, Software, Methodology, Investigation. **Maria Scarasciulli:** Visualization, Software, Methodology, Investigation. **Eleonora Lalle:** Visualization, Software, Methodology, Investigation. **Fabrizio Maggi:** Visualization, Supervision, Methodology, Investigation. **Giulia Piccirilli:** Visualization, Software, Methodology, Investigation. **Tiziana Lazzarotto:** Visualization, Software, Investigation. **Antonio Piralla:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Fausto Baldanti:** Writing – original draft, Supervision, Project administration, Conceptualization. **Elena Pariani:** Writing

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Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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