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RESEARCH ARTICLE

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Molecular characterization of emerging Echovirus 11 (E11) shed light on the recombinant origin of a variant associated with severe hepatitis in neonates

Antonio Piralla[1] | Federica Giardina[2] | Guglielmo Ferrari[1] | Stefano Gaiarsa[1] | Greta Romano[1] | Laura Pellegrinelli[3] | Cristina Galli[3] | Arlinda Seiti[3] | Sandro Binda[3] | Antonino Maria Guglielmo Pitrolo[1] | Angelo Genoni[4,5] | Francesca Drago Ferrante[5] | Federica Novazzi[4,5] | Nicasio Mancini[4,5] | Francesca Rovida[1,2] | Elena Pariani[3] | Fausto Baldanti[1,2]

[1]Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

[2]Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, Pavia, Italy

[3]Department of Biomedical Sciences for Health, University of Milan, Milan, Italy

[4]Laboratory of Medical Microbiology and Virology, Department of Medicine and Technical Innovation, University of Insubria, Varese, Italy

[5]Laboratory of Medical Microbiology and Virology, University Hospital of Varese, Varese, Italy

Correspondence
Antonio Piralla, Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.
Email: antonio.piralla@unipv.it

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Abstract

Echovirus 11 (E11) has gained attention owing to its association with severe neonatal infections. Due to the limited data available, the World Health Organization (WHO) considers public health risk to the general population to be low. The present study investigated the genetic variation and molecular evolution of E11 genomes collected from May to December 2023. Whole genome sequencing (WGS) was performed for 16 E11 strains. Phylogenetic analysis on WG showed how all Italian strains belonged to genogroup D5, similarly to other E11 strains recently reported in France and Germany all together aggregated into separate clusters. A cluster-specific recombination pattern was also identified using phylogenetic analysis of different genome regions. Echovirus 6 was identified as the major recombinant virus in $3C^{pro}$ and $3D^{pol}$ regions. The molecular clock analysis revealed that the recombination event probably occurred in June 2018 (95% HPD interval: Jan 2016–Jan 2020). Shannon entropy analyses, within P1 region, showed how 11 amino acids exhibited relatively high entropy. Five of them were exposed on the canyon region which is responsible for receptor binding with the neonatal Fc receptor. The present study showed the recombinant origin of a new lineage of E11 associated with severe neonatal infections.

KEYWORDS
Echovirus 11 (E-11), enteroviruses, hepatitis, neonatal infection, recombinant strain

1 | INTRODUCTION

An upsurge of severe neonatal cases and fatalities linked to a novel variant of Echovirus 11 (E11) was documented in France, Italy and China.[1–3] World Health Organization (WHO) has gained attention owing to its association with severe neonatal infections since 2022.[4,5] Other cases have been reported occurring in European countries such as Spain, Sweden, and UK.[4,5] However, the prevention and control of E11 variants have been hampered by limited background data on the virus circulation and genetic variance. Additionally, the World Health Organization (WHO), having evaluated the limited data available, considers the public health risk to the general population to be low. Considering that non-polio enterovirus (NPEV) infections are not notifiable infectious diseases in Italy, the circulation of E11 in Lombardy (Northern Italy), where the first two cases of E11 neonatal infection were reported, has been implemented in the period April-December 2023. In detail, an event-based surveillance and a hospital-based surveillance considering inpatients exhibiting respiratory or neurological symptoms have showed a total of 20 additional cases.[6] Recently, a wastewater-based surveillance (WBS) performed in Sicily (a region in Southern Italy) between June 2022 and June 2023, showed that the new E11 lineage has circulated in this region during early 2023.[7] This report together with European data showed a silent unrecognized circulation of this new E11 variant.

The emergence of the novel E11 lineage has been related to recombination events, which apparently allowed this variant strain to infect humans more successfully.[1] However, a complete analysis exploring the parental genomes, genetic variability, and recombinant origin of this emerging variant is still missing. In addition, the increasing pathogenetic role of this variant needs to be fully elucidated with in-vitro models. In the present study, the genetic variation and molecular evolution of the E11 complete genomes through the collection of strains among our surveillances and E11 sequences published in the GenBank database have been investigated.

2 | MATERIAL AND METHODS

2.1 | Sample collection

A total of 16 E11 strains used in this study were from E11-positive patients identified in an event-based surveillance and a hospital-based surveillance considering inpatients exhibiting respiratory or neurological symptoms from May to December 2023. Our analysis aims to describe and examine the circulation of E11 in Lombardy (Northern Italy) as well as include WGS of the first two cases of E11 neonatal infection previously reported in Italy.[2,6] All these samples were analysed in two Lombardy's regional reference laboratories (Microbiology and Virology department, Fondazione IRCCS Policlinico San Matteo, Pavia, and Department of Biomedical Sciences for Health, University of Milan, Milan) as previously reported[2,6] and additional information of clinical samples were reported in Table S1.

2.2 | Next-generation sequencing by metagenomic approach

Total RNA was extracted directly from clinical samples using the QIAsymphony® instrument with QIAsymphony® DSP Virus/Pathogen Midi Kit (Complex 400 protocol (QIAGEN) or QIAamp Viral RNA Mini kit (QIAGEN) by means of an automated extractor (QIAcube, QIAGEN) according to the manufacturer's instructions. RNA was treated with TURBO DNase (Thermo Fisher Scientific, Waltham, MA) at 37°C for 20 min and then purified by RNA Clean and Concentrator-5 Kit (Zymo Research). RNA was used for the assessment of sequencing independent single primer amplification protocol (SISPA) with some modifications reported by Lorusso et al.[8] Libraries were prepared using Nextera DNA Flex Library Prep (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. Sequencing was performed on the MiSeq (Illumina Inc., San Diego, CA) by MiSeq Reagent v2 (300-cycle). The obtained FastaQ were analysed with the CZ ID metagenomic pipeline.[9] The E11 virus consensus sequences were obtained by mapping to the reference with the highest coverage breadth and depth, obtained through the metagenomic pipeline. Accession number of sequences originated in the study are PP498690-PP498703.

2.3 | Phylogenetic analysis and recombinant analysis

All available E11 genomes ($n = 100$ strains) were downloaded from GenBank and used together with the 16 E11 strains originated in this study. Alignment was performed using MAFFT 7.475.[10] Maximum likelihood (ML) trees were constructed in IQ-TREE5[11] with a substitution model chosen according to BIC within the IQ-TREE internal pipeline with 1000 bootstrap replicates. DNA similarity searches of P1, P2, and P3 coding region sequences were performed separately using the NCBI WWW-BLAST (basic local alignment search tool) server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) on the GenBank DNA database (BLAST+ v. 2.15.0). Sequences with >85% similarity were considered prospective parental sequences and retrieved from GenBank. A data set of 15 enterovirus strains (listed in Figure 2 and Table S2) were used for recombination signal screening using SimPlot software (version 3.5.1).[12] A similarity and boot-scanning analysis were performed using a 400-nucleotide sliding window and shifting of 10-nucleotides.

2.4 | Phylodynamic analysis of recombinant genome region

NCBI BLASTn program (https://blast.ncbi.nlm.nih.gov) was used to perform multiple sequence alignments of E-11 new lineage in the $3C^{pro}$-$3D^{pol}$ genome region. Consensus sequences were inspected for nucleotide identity percentage ranging from 85.0% to 100% with a total of 63 sequences used to perform phylogenetic analysis. The

phylodynamic analyses workflow was showed in Figure S1. In detail, MEGA version 11[13] was used to perform the alignment using implemented MUSCLE algorithm and to build the phylogenetic tree based on a Neighbor-joining method with 1,000 bootstrap replicates and a mean nucleotide genetic distance (p-distance). MEGA version 11 and ModelFinder from IQ-TREE v.2.2.2.6[14] were used to select the best nucleotide substitution model (GTR + G + I). The temporal signal of the sequences was investigated using TempEst v.1.5.3[15] to confirm the presence of sufficient genetic change between sampling times ($R^2 = 0.82$, Correlation coefficient = 0.9). A Markov Chain Monte Carlo (MCMC) sampling method, implemented in BEAST v1.10.4,[16] a Bayesian statistical framework, was used to perform phylogenetic analysis and the years of samples collection (retrieved from GenBank) were used to calibrate the molecular clock.

The analyses were performed using an uncorrelated lognormal clock with a constant size model of demographic history. The program default priors on the substitution model (GTR + G) parameters were used in these analyses. Parameter estimates were obtained from MCMC run of 2×10^8 generation and a sampling frequency of 10^3. The performance of the transition kernel was inspected, and the acceptance ratio was greater than 0.234. The posterior distribution for each parameter was visualized with Tracer v.1.7.2,[16] a MCMC trace analysis tool which also estimated the Effective Sample Size (ESS) (i.e., measurement of the number of effectively independent samples in each run) of the parameters sampled from the MCMC. The analysis was considered to have converged and reached stability after the burn-in period when ESS was higher than 200. Maximum clade credibility tree was estimated with TreeAnnotator v1.10.4[16] removing the first 10% of trees as burn-in. Statistical support for the nodes in topology was assessed by a posterior probability (pp) value. The tree was visualized with FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree).

2.5 | Genome mutations analysis

The presence of conserved non-synonymous mutations between the two putative donor genomes E11 (OQ966171) and E6 (OR840838) and the new lineage sequences ($n = 37$ E11 strains) was assessed using the Snipit pipeline (https://github.com/aineniamh/snipit),[17] which was modified to select non-synonymous mutations and to work with amino acid notation.

2.6 | Shannon entropy analysis for measuring diversity

Amino acid variability of the P1 capsid precursor protein (VP4-VP2-VP3-VP1) was assessed using Shannon entropy on the E11 sequences data set ($n = 116$) used for phylogenetic analysis (described above). Shannon entropy was assessed for all 861 amino acids of P1 using an online analysis tool (available at https://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy_one.html),[18] with extremely variable amino acid sites defined as those with entropy values > 0.6. The ChimeraX program[19] was used to visualize the distinct distributions of significant sites based on the 6LA6 model from the Protein Data Bank (PDB) database showing the concealed surface area between the FcRn receptor and capsid proteins (https://www.rcsb.org/structure/6la6).

3 | RESULTS

3.1 | Whole-genome sequence analysis of E11 strains

A total of 16 E11 WGS strains were performed in the present study and included in the analyses. A large open reading frame (ORF) encoding a potential polyprotein precursor of 2195 aa was cleaved into P1 (VP4, VP2, VP3, and VP1), P2 ($2A^{pro}$, 2B, and 2C), and P3 (3A, 3B, $3C^{pro}$, and $3D^{pol}$) regions with 861 aa, 578 aa, and 756 aa, respectively. All available E11 sequences ($n = 100$) retrieved from GenBank were included in a data set used for phylogenetic analysis. The phylogenetic tree showed that all investigated strains belonged to genogroup D5,, similar to other E11 strains recently reported in France and Germany aaccording to Savolainen-Kopra et al., 2009.[20] All aggregated into a separate lineage, including 37 E11 strains (Figure 1A). Within this new lineage, the average nucleotide genetic identity was 98.7% (range 97.9%–100%). Furthermore, nucleotide identity comparisons of WGS demonstrated that the E11 strain, which belonged to a new lineage, had an average of nucleotide genetic identity of 85.1% (range 81.9%–94.2%) compared to other E11 reference strains available in the GenBank database ($n = 79$ strains). A cluster-specific recombination pattern was also identified using phylogenetic analysis of the P1, P2, and P3 genome regions, and was analysed separately (data not showed). In the P1 and P2 capsid coding regions, the new lineage strains clustered together with the strain PMB_230005716902_FRA_2023 (OQ969171, Figure 1B). In the tree based on the P3 sequences, the new lineage strains clustered outside the E11 tree as an outgroup (data not showed). BLAST analysis of the P3 region sequence showed a high nucleotide identity >95% with the Echovirus 6 strain EV6_Fr22_-MAR9310 (OR840838 published in GenBank on December 18, 2023) suggesting the occurrence of one putative recombination event.

3.2 | Recombination and evolutionary analyses

BLAST results combined with the phylogenetic trees reported in Figure 1 showed that E6 was identified as the major putative parent for $3C^{pro}$ and $3D^{pol}$ regions (Figure 1C). SimPlot software was used to determine the recombination site position using all the 37 E11 strains, belonged to the new lineage, as query sequences (Figure 2A). The SimPlot results confirmed that the E11 strains had the highest similarity with the E11 (OQ969171) prototype strain in the P1, P2 and 3A-3B regions. Whereas in the $3C^{pro}$ and $3D^{pol}$ regions, the

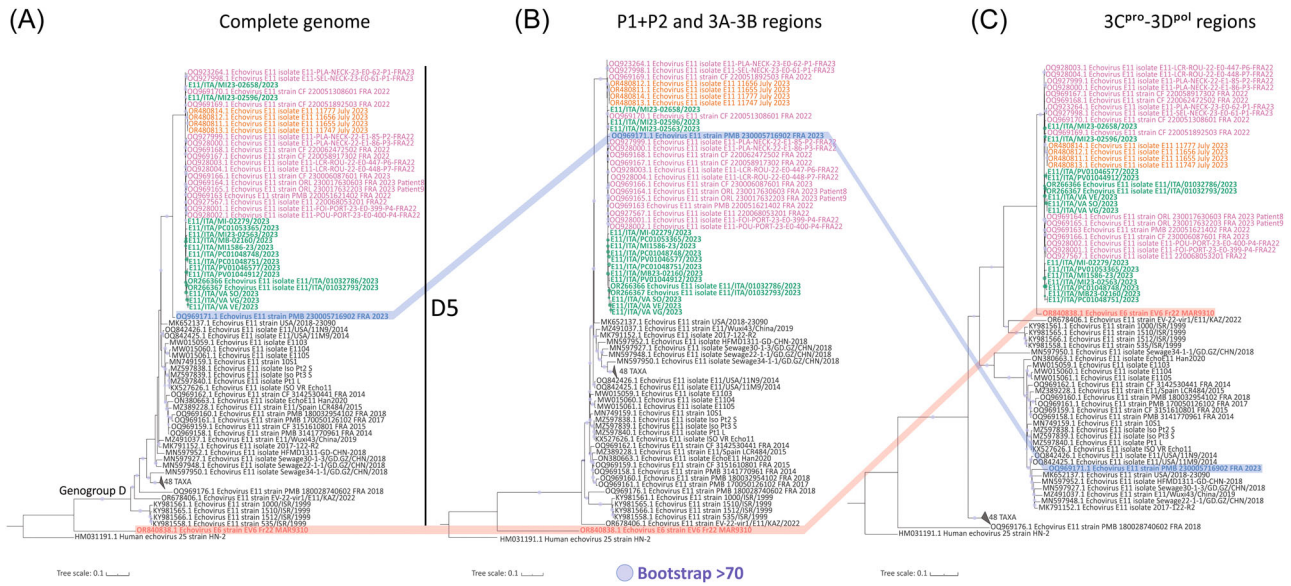

FIGURE 1 ML Phylogenetic trees constructed based on (A) complete genome (B) P1-P2 and 3A-3B (C) $3C^{pro}$-$3D^{pol}$ genome regions. Italian E11 strains ($n = 16$) are colored in green and their most related enterovirus sequences are reported in cerulean (E11, OQ696171) and coral (E6; OR840838), respectively. All available E11 strains belonged to new lineage ($n = 17$ from France[1] reported in purple and $n = 4$ from Germany reported in orange) are included. Scale bars represent the replacement of each site per year.

highest similarity score was observed with the E6 strain (OR840838). The phylogenetic trees established with the selected sequences (Figure 1B and C) were consistent with the results of SimPlot (Figure 2A) and BootScanning (Figure 2B), which confirmed the results of the recombination analysis.

To establish the timing of the recombination event, a phylodynamic analysis was performed using an alignment of multiple sequences of the $3C^{pro}$ and $3D^{pol}$ regions based on similarities obtained from BLAST analysis. The topology inferred by Beast package using the best fit models was used to describe the phylogenetic relationships that better characterized the samples of E11 new lineage strains and E6 as the parental genome of the $3C^{pro}$ and $3D^{pol}$ regions. The Bayesian molecular clock analysis was performed to infer the MCC tree and presented in Figure 3. The Bayesian analysis estimated a mean evolutionary substitution rate of 6.32×10^{-3} subs/site/years (95% HPD interval: 4.31×10^{-3}–8.57×10^{-3}). The molecular clock analysis revealed that the recombination event probably occurred in June 2018 (95% HPD interval: Jan 2016–Jan 2020; Figure 3), also assuming the common ancestor for E11 (new lineage) and E6 strains (OR840838) in the $3C^{pro}$ and $3D^{pol}$ regions.

3.3 | Genetic variance of genome

To identify crucial amino acid mutation sites that may affect the virulence of the variants, the modified Snipit script (https://github.com/aineniamh/snipit) was used to visualize the relative changes of each amino acid site as compared to the most related strains. A total of 102/2195 (4.6%) amino acid positions were observed to have at least one change in the coding sequences alignment as compared to parental genomes (E11, OQ696171 and E6, OR840838) (Figure 4). Of these, 40/102 (39.2%) changes occurred within the P1 region (structural proteins), 28/102 (57.5%) within the P2 region, 8/102 (7.8%) within the 3A-3B region and 26/102 (25.5%) in the recombinant $3C^{pro}$ and $3D^{pol}$ regions. Among the changes of the P1 region, 21/40 (52.5%) are located within the VP1 protein but none of these changes were in the BC- and DE-loop. A total of 11 amino acid changes were fixed in all the E11 strains belonged to the new lineage.

An alignment of P1 sequences was also examined using the Shannon entropy online analysis tool. A total of 11 amino acids (VP2, 136 and 138; VP3, 35, 64, 135, and 234; VP1, 92, 144, 235, 262 and 268) exhibited relatively high entropy values (higher than 0.60), suggesting a notable degree of amino acid diversity (Figure 5A). Cryo-electron microscopic structure of E11 and FcRn was available and used to point out results of entropy analysis (Figure 5B). Seven amino acids selected were exposed on the surface of the capsid on a canyon region responsible for receptor binding and colored in white in the 3D model (Figure 5B).

4 | DISCUSSION

The evolution history of E11 was well characterized since the 2004 by the phylogenetic analysis on E-11 isolates identifying several genogroups named A, B, C, and D1–D5.[21] The monitoring of E11 evolution confirmed the prevalence of genogroup D5 with distinct strains in the last 15 years.[20,22] Since summer 2022, a divergent lineage of E11 belonging to genogroup D5 has recently been associated with an increased number of hepatitis episodes in

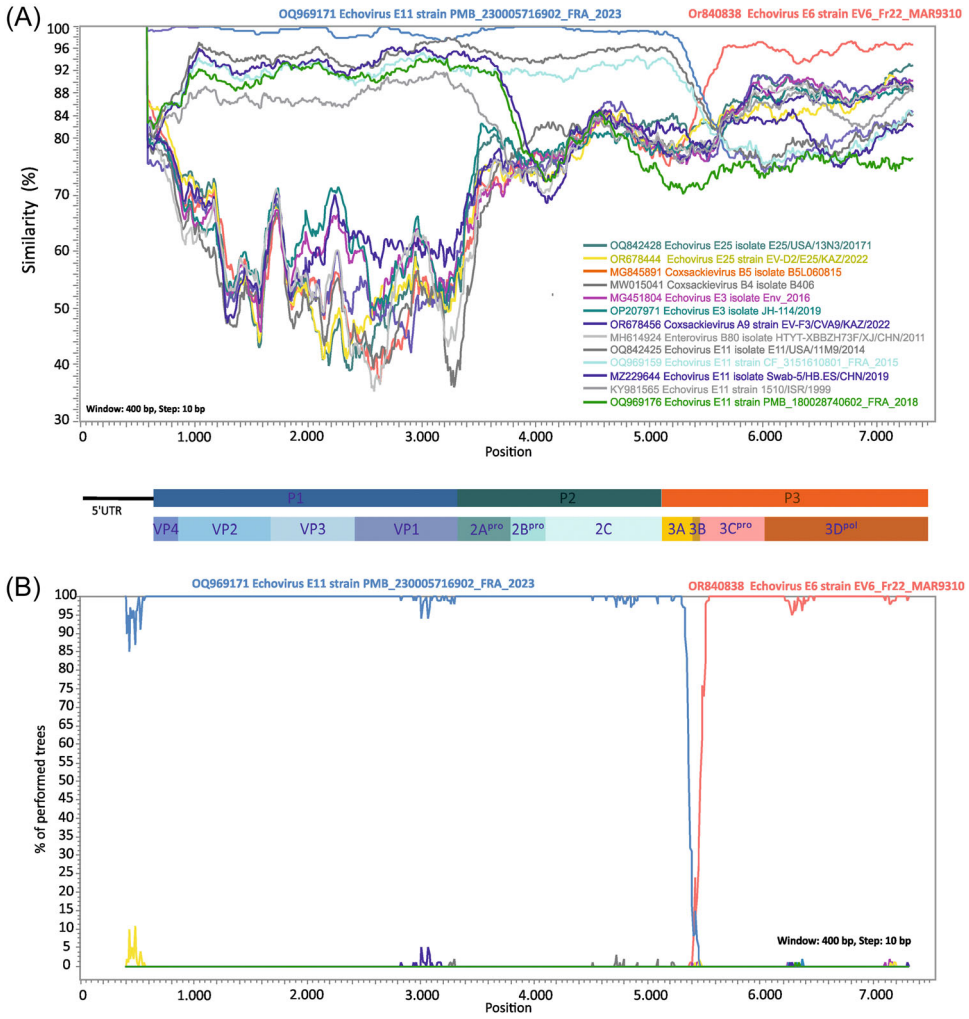

FIGURE 2 Recombination analyses of the E11 new lineage strains used as query ($n = 37$; $n = 16$ Italian, $n = 17$ from France[1] and $n = 4$ from Germany) with other EV-B strains. (A) Similarity plots and (B) boot scanning analyses of strains with potential parents.

neonates in Europe.[4] Since 2018, severe neonatal infections and mortality associated with genogroup D5 of E11 have been reported in Taiwan, and in the Hubei, and Guangdong provinces of China.[23,24] In addition, this scenario has also been described in a retrospective study in China reporting data from an E11 outbreak occurred in 2019.[3] In this report, severe infections defined as hepatic dysfunction or liver failure were observed in 30 out of 105 (28.6%) neonates.[3] However, a recently commentary of this study raises a concern about the results of those and potential new studies, if genomic data will be used to draw conclusions on association of E11 and unexpected clinical picture.[25]

The main medical needs emerged from a general point of view is the lack of notifiable status for enterovirus infections in many EU countries, thus there may be more cases than are currently known. This observation is directly linked to the unrecognized clinical presentations of the great majority of enterovirus infections. Data from previous studies suggest that more than 90% of patients with E11 infection are asymptomatic or present with mild fever.[26] Additionally, some enteroviruses may remain undetected for years before suddenly reappearing.[27] The lack of a notifiable disease status has resulted in low awareness among healthcare providers, possibly leading to an underdiagnosis of emerging enterovirus variants. An additional value could be obtained through wastewater surveillance, which can provide an early warning of viral spread in communities and offer crucial information about virus circulation and prevalence, as currently utilized for poliovirus.[28] In this perspective, an increased detection rate of E11 belonging to the new lineage in wastewater samples since August 2022 in the Sicily region (Southern Italy) has recently been reported.[7] These findings suggest that WBS is an important tool for enterovirus surveillance to promptly detect the emergence or re-emergence of variants that warrant public health control measures.

Phylogenetic analysis of WGS showed the spread of a monophyletic lineage including E11 strains identified in Italy, France, and Germany.[1,2,4] However, many other EU countries, such as Croatia, Spain, Sweden, and the UK have reported E11 cases during the 2022-2023 period but still no sequences are currently available.[4] Despite the limited public health impact assigned by the WHO to this

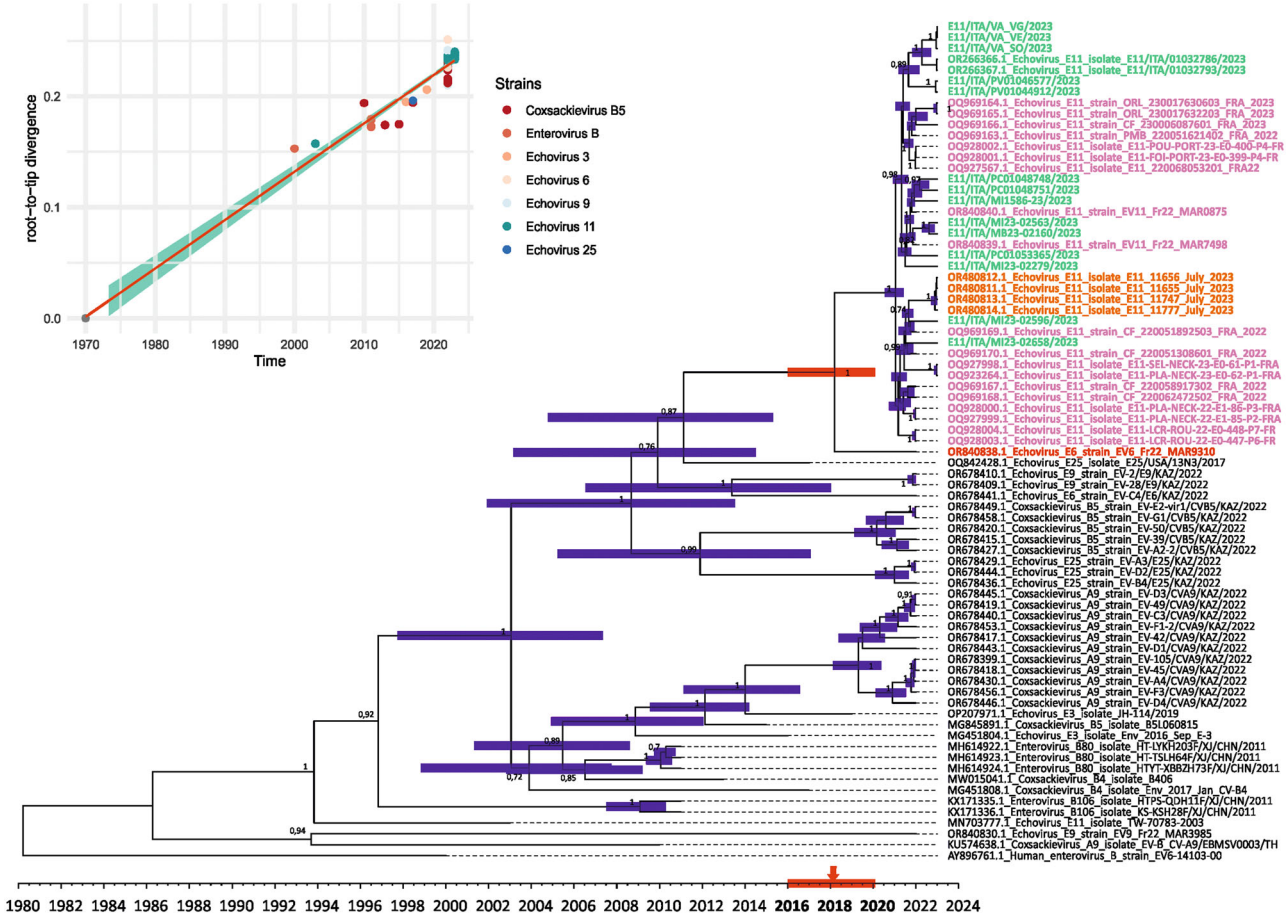

FIGURE 3 The MCC phylogenetic tree was generated using the MCMC method based on $3C^{pro}$-$3D^{pol}$ nucleotide sequences of Enterovirus strains with blast nucleotide identity results >85%. The blue bars indicate the 95% highest posterior density (HPD) for ancestor estimates. The HPD estimation of recombinant event is reported with a red bar. The *x*-axis is the time scale (years).

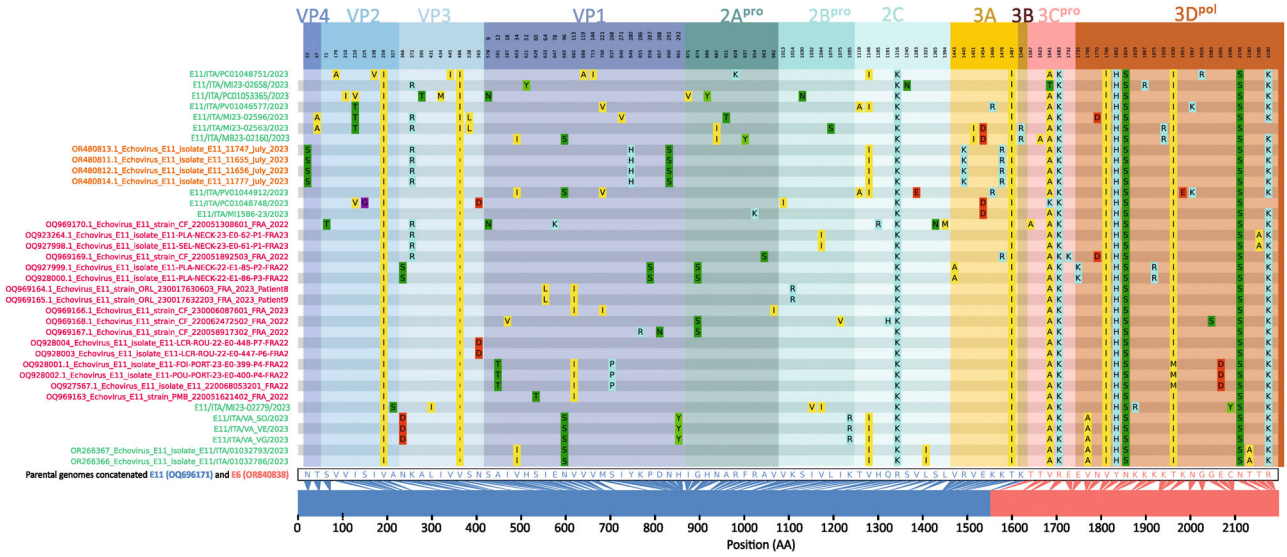

FIGURE 4 The figure was rendered using the *Snipit* tool and modified pipeline for amino acid visualization. (https://github.com/aineniamh/snipit). Italian E11 strains are colored in sea green. All available E11 strains belonged to new lineage (n = 17 from France[1] reported in purple and n = 4 from Germany reported in orange) are included.

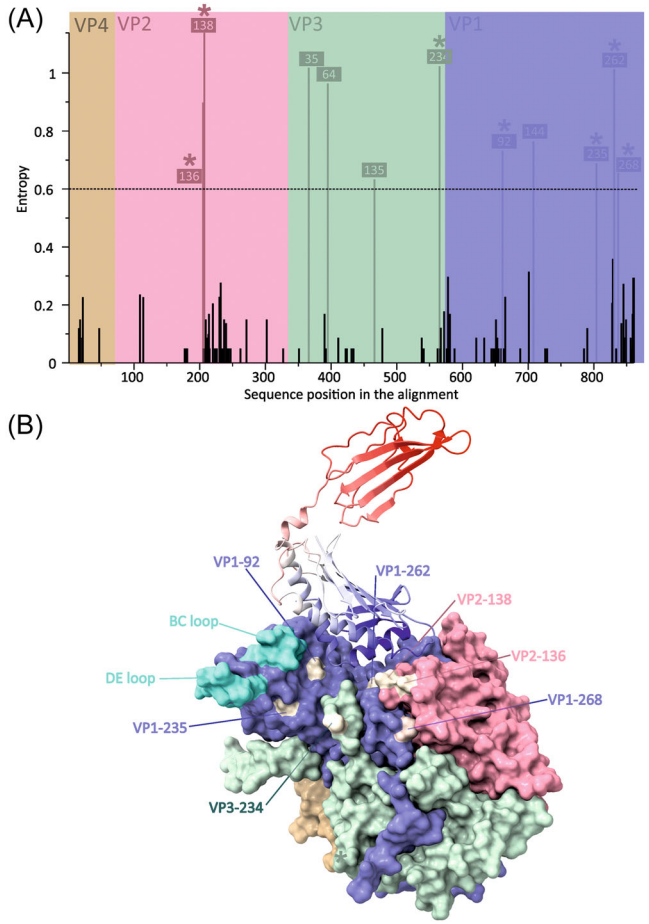

FIGURE 5 Analysis of the genetic variance of all available E11 sequences (n = 116). (A) Entropy value analysis of all 861 amino acids of the P1 region with a threshold value of 0.6. Position of amino acids with value > 0.6 are reported with a box. (B) 6LA6 as a model to illustrate the distribution of each amino acid site in E11 virus particles. The proteins are colored by chains: VP1 (blue), VP2 (rose gold), VP3 (green), VP4 (yellow). The potential interaction between amino acid sites and the FcRn receptor is also showed in the model. BC and DE loops are highlighted in cyan. Amino acids selected by entropy analysis and exposed at the surface of the 3D model and colored in white and reported with an asterisk in the panel A.

new E11 lineage, a consolidated surveillance system needs to be further implemented.[4] A phylogenetic incongruence between structural and nonstructural genes was observed suggesting that recombination events occurred by Grapin et al.[1] However, similarity as well as bootscan analyses presented in the French study were performed without the parental genome. In our study, the recombinant origin hypothesized by Grapin et al[1] was proved because on December 18th, 2023 was deposited in the public GenBank database a WGS of E6 strain (OR840838, Echovirus E6 strain EV6_Fr22_-MAR9310), identified in France in 2022. Therefore, further analyses using different approaches such as phylogenesis and algorithms on sequence similarity and detecting recombination (Bootscanning) revealed that E6 was the parental donor of the $3C^{pro}$ and $3D^{pol}$ genome regions for the new E11 lineage. Recombination is a well-known mechanism for enteroviruses evolution as previously observed for E11 as well.[29–31] However, none of these reports showed recombination in the $3C^{pro}$ and $3D^{pol}$ regions. More precise identification of the individual recombination events in E11 was achieved using time-correlated tree analysis and superimposition of branching points in the $3C^{pro}$ and $3D^{pol}$ tree, also previously used for EV-71.[32] The tMRCA of the new lineage of E11 and E6 viruses was dated between 2016 and 2020, suggesting a silent circulation of E11 until its emergence in 2022. This finding is also supported by the evolutionary rate calculated for E11, which is congruent with NPEV substitution rates estimated by others.[32,33]

Amino acid changes in structural proteins may affect viral virulence, as well described for NPEV.[34] Sequence comparison of the P1 region in the present study identified several positions with a significant Shanonn entropy not only located in the VP1 region. A series of seven (VP2, 136 and 138; VP3, 234; VP1, 92, 235, 262 and 268) amino acids with increasing entropy are neighboring to the "canyon" of the receptor-binding regions inside the monomeric structure of the capsid. This is a key area governing the binding of the FcRn receptor.[35] These mutations occurring at this specific site might affect the binding and the uncoating process of E11 and, thus, increase its transmission ability. None of the selected positions were situated in the critical binding regions for neutralizing antibodies that correspond to the BC or DE loop within the VP1 protein. Mutations in these epitopes have been associated with the virus ability to evade the immune system.[36] Although several specific amino acid mutations were observed in the present study, their significance requires further investigation. The impact of recombination on the virulence or pathogenesis of the new E11 lineage is currently undefined and needs further experimental investigation. In many cases other factors other than the genetic backbone of E11 could have driven the severity of these infections such as premature birth, lack of maternal immunity and the young age.

In conclusion, the present study showed the recombinant origin of a new lineage of E11 associated with severe neonatal infections. Further studies aiming at elucidating the increased pathogenicity of E11 variant are needed to better correlate genetic information with unexpected clinical presentations. WGS of enteroviruses is needed to evaluate the presence of recombinant strains and to better evaluate the phylodynamic and phylogeography in the context of molecular epidemiology of emerging enterovirus variants.

AUTHOR CONTRIBUTIONS

Antonio Piralla wrote the manuscript and design the study and experiments. Federica Giardina, performed most of the experiments with help from Guglielmo Ferrari, Antonino Maria Guglielmo Pitrolo, Laura Pellegrinelli, Cristina Galli, Arlinda Seiti, Angelo Genoni, Francesca Drago Ferrante, and Federica Novazzi. Stefano Gaiarsa, Greta Romano performed bioinformatic analysis; Sandro Binda, Nicasio Mancini, and Francesca Rovida contributed to the discussion and provided reagents., Elena Pariani, Fausto Baldanti supervised the overall study and reviewed the manuscript and provided resources. All of authors helped with data analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article. The data that support the findings of this study are available in the supplementary material of this article. Sequence data generated in this study have been submitted to GenBank under accession numbers PP498690-PP498703.

ETHICS STATEMENT

Ethical review and approval were waived for this study, considering that the study was conducted on referred samples as part of the routine activities of the Italian influenza and other respiratory virus surveillance network (InfluNet & RespirVirNet) and the routine management and treatment of patients.

ORCID

Antonio Piralla http://orcid.org/0000-0002-6062-2579
Laura Pellegrinelli http://orcid.org/0000-0003-2344-6032
Francesca Drago Ferrante http://orcid.org/0000-0003-4950-0703
Fausto Baldanti http://orcid.org/0000-0002-3358-8969

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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