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Original Research

Genetic characterizations and molecular epidemiology of human echovirus 30 isolated from Ningxia, China

Fang Yuan [a,1], Xinfeng Wei [b,1], Xueping Ma [a], Jiangtao Ma [a], Xuemin Ma [a], Xiaoqiang Sun [a], Min Cao [a], Juan Zhou [a], Wei Zhang [a], Hui Chen [a], Rui Wang [c], Jichen Li [c], Qiang Sun [c,*]

[a] Ningxia Hui Autonomous Region Center for Disease Control and Prevention, Yinchuan 750004, China
[b] Ningxia Hui Autonomous Region People's Hospital, Yinchuan 750021, China
[c] National Laboratory for Poliomyelitis, WHO WPRO Regional Polio Reference Laboratory, National Key Laboratory for Traceability, Early Warning and Intelligent Decision of Infectious Diseases, National Health Commission Key Laboratory for Biosafety, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China

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ABSTRACT

In October 2017, a small outbreak of echovirus 30 (E30) associated with aseptic meningitis in nine cases occurred at a primary school in the Ningxia Hui Autonomous Region. That year, we observed a significant increase in E30 levels in an acute flaccid paralysis (AFP) case surveillance system. To investigate their phylogenetic relationships, we determined the whole genomic sequences of 12 strains isolated from aseptic meningitis cases, AFP cases, and healthy children. We found that the E30 strains circulating in Ningxia belong to two lineages (H and J). The strains isolated in 2010, 2012, and 2016 belonged to the H lineage. In 2017, a new lineage, J, emerged as the dominant lineage. Phylogenetic trees were constructed based on the whole genome and *P1*, *P2*, and *P3* regions; clustering with other types of enterovirus species B was found, suggesting that recombination events had occurred. The recombination sites were mainly in regions *2B*, *2C*, and *3D*. This study confirmed that the E30 strains in Ningxia in 2010, 2012, and 2016 had different recombination patterns and were recombined with different enteroviruses. The 2017 epidemic E30 originated from another new lineage with a complex recombination pattern and formed an independent transmission chain in Ningxia.

1. Introduction

Echovirus 30 (E30) is a human enterovirus species B (EV-B) serotype within the Picornaviridae family. The clinical manifestations of E30 range from asymptomatic to mild febrile illness, all the way to severe central nervous system diseases such as encephalitis, aseptic meningitis (AM), and acute flaccid paralysis (AFP) [1–4]. E30 is the most common cause of viral meningitis worldwide. In Europe, E30 is the most frequently detected genotype among the common enteroviruses and is most frequently detected in neurological infections and cerebrospinal fluid [5–7]. It is often the cause of large epidemics, closely related to the emergence of new lineages [8]. In China, E30 reports have mainly focused on meningitis outbreaks in many provinces, as E30 infections have begun to occur mainly in densely populated eastern coastal areas such as Zhejiang, Jiangsu, Fujian, Shandong, and Guangdong Province [9–12]. In recent years, the transmission of E30 has been reported in Shanxi, Henan, Inner Mongolia Autonomous Region, and other provinces or cities [13,14]. Studies have shown that E30 migrates gradually from coastal to inland areas in China [14].

The Ningxia Hui Autonomous Region is in western China, neighboring the Inner Mongolia Autonomous Region and Gansu Province. Before 2017, E30 was rare in Ningxia; only three strains had been isolated from the AFP case surveillance system, which has been continuously monitored in the region for over 20 years. In 2017, we observed a sudden increase in E30: five strains were isolated from the AFP case surveillance system, and four strains were isolated during an AM outbreak in October. The relationship between these different sources of E30 and the different observed diseases is still unknown, and few relevant studies are available covering E30 molecular genetic variation. The biological mechanisms of virulence and adaptation of different E30 lineages remain unclear; however, we must understand them better to control outbreaks and design targeted prevention and treatment strategies. In this study, we determined the complete nucleotide

* Corresponding author: National Laboratory for Poliomyelitis, WHO WPRO Regional Polio Reference Laboratory, National Key Laboratory for Traceability, Early Warning and Intelligent Decision of Infectious Diseases, National Health Commission Key Laboratory for Biosafety, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China.
E-mail address: sunqiang8611@126.com (Q. Sun).
[1] These authors contributed equally to this work.

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HIGHLIGHTS

Scientific question

Echovirus type 30 (E30) is a human enterovirus species B (EV-B) serotype that can cause severe central nervous system diseases such as encephalitis, aseptic meningitis (AM), and acute flaccid paralysis (AFP). Novel E30 variants have invariably undergone recombination with other EV-B types before their emergence. Studying the recombination model of novel E30 variants is crucial.

Evidence before this study

Before 2017, E30 was rare in Ningxia Hui Autonomous Region. In 2017, we observed a sudden increase in E30: five strains were isolated from the AFP case surveillance system, and four strains were isolated during an AM outbreak in October. To understand the cause of the sudden increase, the whole genome sequence of the virus was performed to understand its gene characteristics and its relationship to the disease.

New findings

In this study, we found that the E30 strains circulating in Ningxia belong to two lineages (H and J). This study confirmed that the E30 strains in Ningxia in 2010, 2012, and 2016 had different recombination patterns and were recombined with different enteroviruses.

Significance of the study

The 2017 epidemic E30 originated from another new lineage with a complex recombination pattern and formed an independent transmission chain in Ningxia.

sequence of E30 from different cases in Ningxia to investigate the genetic relationships between these isolates and determine their molecular characteristics and phylogenetic relationships with other strains of EV-B. We aimed to define the genetics of these isolates, understand the molecular variation and genetic evolution of the E30 genome in Ningxia, explore its relationship with the outbreak, and provide a reference for preventing and controlling future epidemics.

2. Materials and methods

2.1 Source of the isolates

Clinical samples were collected using the AFP case surveillance system, and viruses were isolated from stool samples cultured in human rhabdomyosarcoma (RD) cells. The samples were processed and inoculated according to the WHO polio laboratory manual for poliovirus (PV)/enterovirus (EV) isolation. Three strains were isolated in 2010, 2012, and 2016, respectively. In 2017, five more strains were isolated from healthy children with AFP. In October 2017, 10 cases of aseptic encephalitis occurred in two primary schools in Xi Xia District, Yinchuan City, in Ningxia. A total of 20 specimens of pharyngeal swabs and cerebrospinal fluid from this outbreak were sent for examination, resulting in another four strains being isolated. Specific information is listed in Table 1.

2.2. Nucleic acid extraction and entire VP1 region amplification

Viral RNA was extracted using a commercial RNA extraction kit (BioPerfectus technologies, Jiangsu, CHN) according to the manufacturer's instructions. Using a one-step reverse transcript-polymerase chain reaction (RT-PCR) kit (TransGen Biotech, Beijing, CHN) to amplify the entire *VP1* region of E30, according to the protocols of previous studies [13]. The primer sequence used for PCR amplification is E30-VP1-S (CCTACACTGATGCGGGCTRY) and E30-VP1-A: (CAACGTGTGTAGCGAGGTGR). Positive PCR products were then sequenced, analyzed by the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov), and genotyped using the Enterovirus Genotyping Tool (version 0.1) [15].

2.3. Whole-genome sequence determination

The whole genome was sequenced using the Illumina platform. BioGerm Nucleic Acid Extraction and Purification Reagent (Berger TQ-BG-001-96B) was used to extract the nucleic acids. The enterovirus RNA was first reverse-transcribed into cDNA using the Super Script III First-Strand Synthesis System for RT-PCR kit from ThermoFisher Scientific Inc. (Waltham, MA, USA) and stored in a -20℃ refrigerator. Capture was performed using the enterovirus genome enrichment kit (Shanghai Berger Biotechnology Co., Ltd.). The Illumina TruSeq Nano DNA LT Library was constructed using the Illumina TruSeq DNA Sample Preparation Guide. The mixed samples (10 nmol/L) were diluted and quantified step-by-step to 4-5 pmol/L and then sequenced using Illumina NextSeq. After sequencing, the viral genomic sequence was assembled using SPAdes v3.13.2 software [16].

2.4. Analysis of bioinformatics software

After alignment with ClustalW, maximum likelihood phylogenetic analysis was performed using MEGA X for the *VP1* region sequences and whole-genome sequences inferred by bootstrapping for 1,000 replicates [17]. Recombination event analysis was performed using Simplot software (version 3.5.1) for recombination analysis between E30 and other EV-B endemic strains [18]. The analysis parameters were: Kimura (2-parameter) and Neighbour-Joining alternative models, 20 bases spacing (step: 20 bp), 200 nt sequence fragment delineation (window: 200 bp), nucleotide transition and transversion rate ratio of 2.0. The recombination detection program RDP4 (v.4.46) [19] automatically identified specific breakpoint locations using seven methods: RDP4, GENECONV, MaxChi, Bootscan, Chimaera, SiScan, and 3Seq. In RDP4, every possible recombination site in the E30 genome was examined, looking at a window size of 100 nt under default parameters.

3. Results

3.1. Clinical findings

The clinical features of the two AFP cases started with a fever and presented with symptoms of flaccid paralysis, but the location of the paralysis was different. Case P10039 was upper limb paralysis, while case P12022 was left arm and left leg paralysis accompanied by sensory disorders in the limbs. The two AFP patients had neck stiffness and muscle pain, but the muscle tone was normal. They all showed no residual paralysis during a 60-day follow-up.

All 10 aseptic meningitis cases were male, aged 10-12 years. The interval between the first case and the last case is nine days. The most common symptoms of patients were headache (100%), fever (100%), repeated vomiting (70%), drowsiness (50%), and dysphoria (50%), but there was no diarrhea. One case presented with abdominal pain, and ultrasonography showed multiple lymphadenites in the lower right abdomen. The first case presented with severe symptoms and hyperspasmia occurring four times within one day and multiple hyperspasmia during hospitalization. The meningeal irritation sign was positive. All patients received cerebrospinal fluid examination, and the

Table 1
Epidemiological and clinical information of E30 strains in this study.

Isolation	Sampling date	Cases	Specimen	Age, years	Sex	Location	Clinical symptoms
P10039	2010.10	AFP	Feces	4	Female	Zhongwei	Paralysis, headache, fever
P12022	2012.08	AFP	Feces	3	Female	Yuanzhou	Paralysis, headache, fever
C16119	2016.12	HC	Feces	3	Female	Yinchuan	No
C17006	2017.01	HC	Feces	5	Male	Tongxin	No
C17052	2017.07	HC	Feces	5	Female	Xiji	No
C17053	2017.07	HC	Feces	3	Male	Xiji	No
C17054	2017.07	HC	Feces	5	Female	Xiji	No
C17099	2017.11	HC	Feces	2	Female	Qingtongxia	No
NY17006	2017.09	AM	Rectal swab	10	Male	Yinchuan	Dizziness, headache, vomiting, dysphoria
NY17007	2017.09	AM	Rectal swab	11	Male	Yinchuan	Dizziness, headache, fever, vomiting, drowsiness
NY17008	2017.09	AM	Rectal swab	10	Male	Yinchuan	Dizziness, headache, fever, vomiting, drowsiness, dysphoria
NY17009	2017.09	AM	Rectal swab	12	Male	Yinchuan	Dizziness, fever, abdominal pain, diarrhea, convulsions, Drowsiness

Abbreviations: E30, echovirus 30; AFP: acute flaccid paralysis; HC: healthy children; AM: aseptic meningitis.

examination results were mild to moderate leukocytosis and monocyte. The results of electroencephalography and head CT were standard in all patients. Except for the first case, all patients' symptoms disappeared after two days of treatment.

3.2. The nucleotide similarity analysis

The full length of the 12 E30 strains examined in this study contained the complete coding sequence of the polyprotein, as well as the 5′ UTR and 3′ UTR portions of between 7,427 and 7,429 bp. The Ningxia strain's 12 E30 full-length nucleotide sequence had low similarity with the 'Bastiann' (AY302558) prototype strain, at 82.0%-82.3% identity. The nucleotide sequence similarity of the 12 E30 isolates was 91.7%-100%. The nucleotide similarity of the nine isolates from 2017 showed high similarity, at 99.7%-100% identity; the similarity between the three strains sampled in 2010, 2012, and 2016 was 95.6%-97.3%; and the similarity between the isolates from 2010 to 2016 and the isolates from 2017 with other E30 from before 2017 was 91.7%-92.7%. The highest homologies to the 2017 strains from this study were from the Inner Mongolia Autonomous Region of China (MW080377, MW080374) at 95.8%-96.0%, a 2017 US strain (MK238483) at 93.9%-94.1%, and a 2017 sample from New Zealand (MW586892) at 97.1%-97.4%.

3.3. E30 Phylogenetic analysis

To investigate the relationship among the E30 strains detected in Ningxia, 50 available entries of the *VP1* sequence in GenBank were compared with homologs from different countries (Fig. 1). According to the phylogenetic classification of the ten lineages (A-J) [9], sequences from 2010 (P10039), 2012 (P12022), and 2016 (C16119) belonged to the 'lineage H,' which included sequences from Guangdong, Zhejiang, and Gansu Province. Notably, the C16119 sequences were closely related to those from Gansu collected in 2016. The sequences from 2017 belonged to a new 'lineage J' genealogy, and this lineage included sequences from Inner Mongolia, the United States, and New Zealand, indicating a wide range of genealogical prevalence. Phylogenetic trees were constructed by aligning the sequences of the complete EV-B genomes and coding regions *P1*, *P2*, and *P3* of the Ningxia E30 strains with the EV-B strains available in the GenBank database (Fig. 2) to investigate the relationship between the E30 strains and other EV-B groups. The complete genome and other EV-B genomes showed that all E30 forms of Ningxia formed a lineage closely related to the E30 representative strain (DQ534205). One study isolates formed a cluster with all of the E30 strains of echovirus 25 (E25) (KX19460). In the *P1* capsid coding region, E30 formed a close-clustering lineage with E30 (DQ534205), E25 (KX19460), and echovirus 21 (E21) (AY302547). The phylogeny of the *P2* and *P3* regions showed different results: E30 in this study was interspersed with other EV isolates but not with the prototype Bastiann strain. The *P2* region was clustered closer to the echovirus 12 (E12) prototype strain (X79047), coxsackievirus A9 (CVA9) (KP290111), and echovirus 11 (E11) (MN496161). The *P3* non-capsid region clustered closest to EV-B prototype strains such as enterovirus B87 (EV-B87), enterovirus B88 (EV-B88), and enterovirus B111 (EV-B111). These results suggest that recombination events probably occurred.

3.4. Recombination analysis

Recombination analysis was performed using Simplot software version 3.5.1 on the sequences of this study and other enterovirus genotypes of the EV-B downloaded from GenBank to confirm whether recombination events had occurred. Similarity plots and boot scanning analysis showed that the isolates in this study had undergone potential recombination events with the representative strain of EV-B in both the 2A-2B linkage region and the *3D* region (Fig. 3). The whole genomes of E30 and other major representative strains of EV-B viruses in this study (based on the more closely-related sequences in the evolutionary tree of the *P1*, *P2*, and *P3* regions) were analyzed for recombination using seven methods (RDP, GENECONV, MaxChi, Bootscan, Chimaera, SiScan, and 3Seq) in RDP4 recombination software. A whole-genome sequence recombination analysis was performed to screen for recombination signals. RDP, GENECONV, Boot Scan, Max Chi, Chimaera, Si Scan, and 3Seq detected several recombination signals. In particular, many recombination signals were detected in the *P2* region, most similar to the CVA9, E25, and coxsackievirus B4 (CVB4). The *P3* region showed the highest similarity with E11 and E6. P10039, P12022, and C16119 contained many recombination breakpoints in the *2C* region, which occurred in the *2B* and *2C* regions with echovirus 6 (E6), echovirus 11 (E11), E25, and CVA9. (Fig. 4).

4. Discussion and conclusion

Since the start of the AFP case surveillance program, E30 has been rare in Ningxia and has only been detected in a few years. However, in 2017, five E30 strains were isolated during AFP case surveillance, with dispersed temporal and regional distribution, from young children aged 2-5 years. By contrast, during a small meningitis outbreak in an elementary school in October of the same year, nine children were infected in the age group of 9-12 years, suggesting that E30 spread silently in the population before the meningitis outbreak. Around the same period, Gansu and Inner Mongolia, which are immediately adjacent to Ningxia, also reported large-scale meningitis outbreaks caused by E30 in 2015 and 2016, respectively [13,14]. Both of these outbreaks involved higher numbers of cases and wider ranges but were consistent with the Ningxia outbreak regarding age distribution and

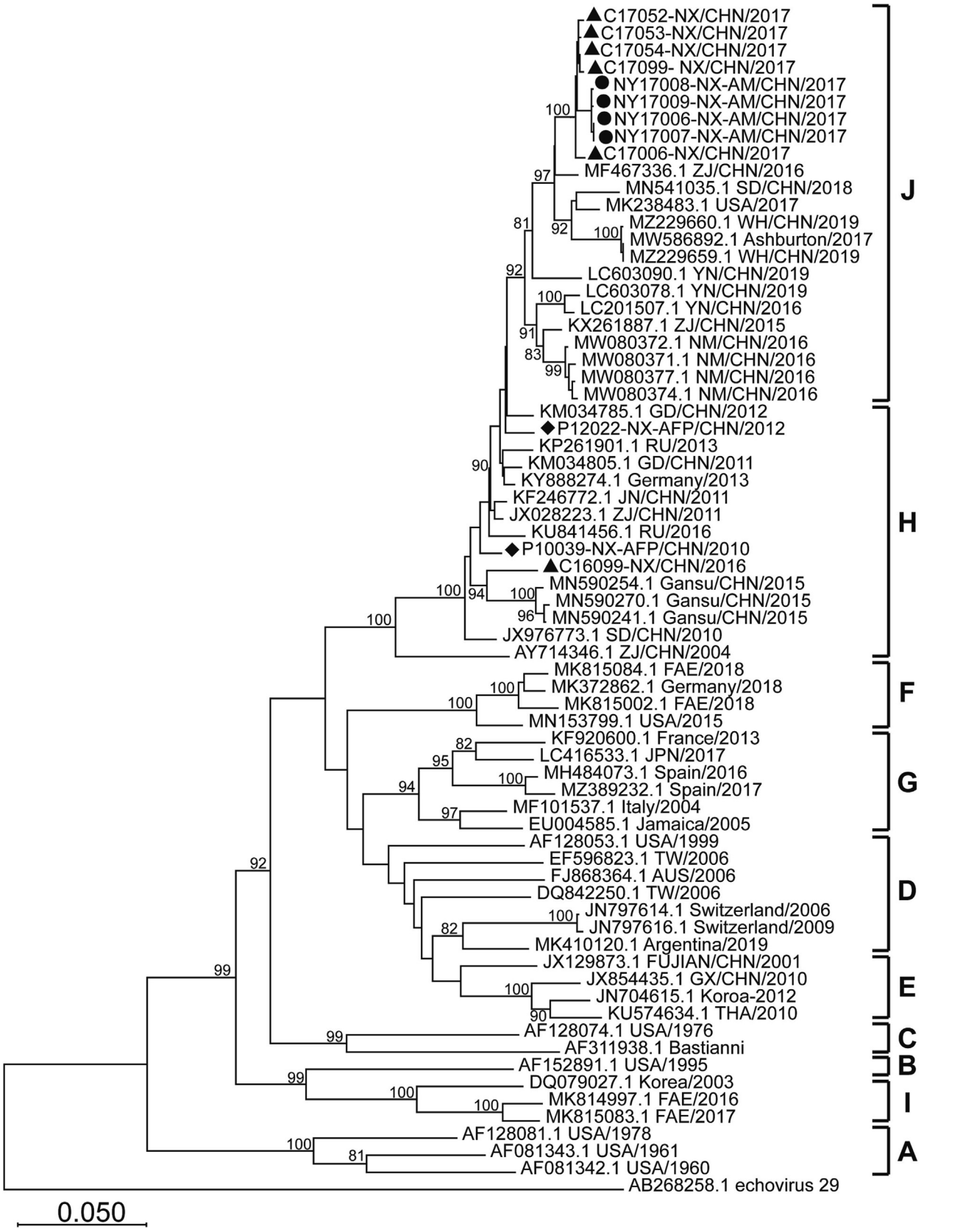

Fig. 1. Maximum-likelihood phylogenetic tree based on entire *VP1* sequences (876 nt) from echovirus 30 strains, inferred by bootstrapping for 1,000 replicates. Triangles represent strains isolated from healthy contacts, solid circles represent strains isolated from acute meningitis cases, and diamonds represent echovirus 30 (E30) isolated from acute flaccid paralysis cases. Echovirus 29 (E29) was used as an outgroup to root the tree.

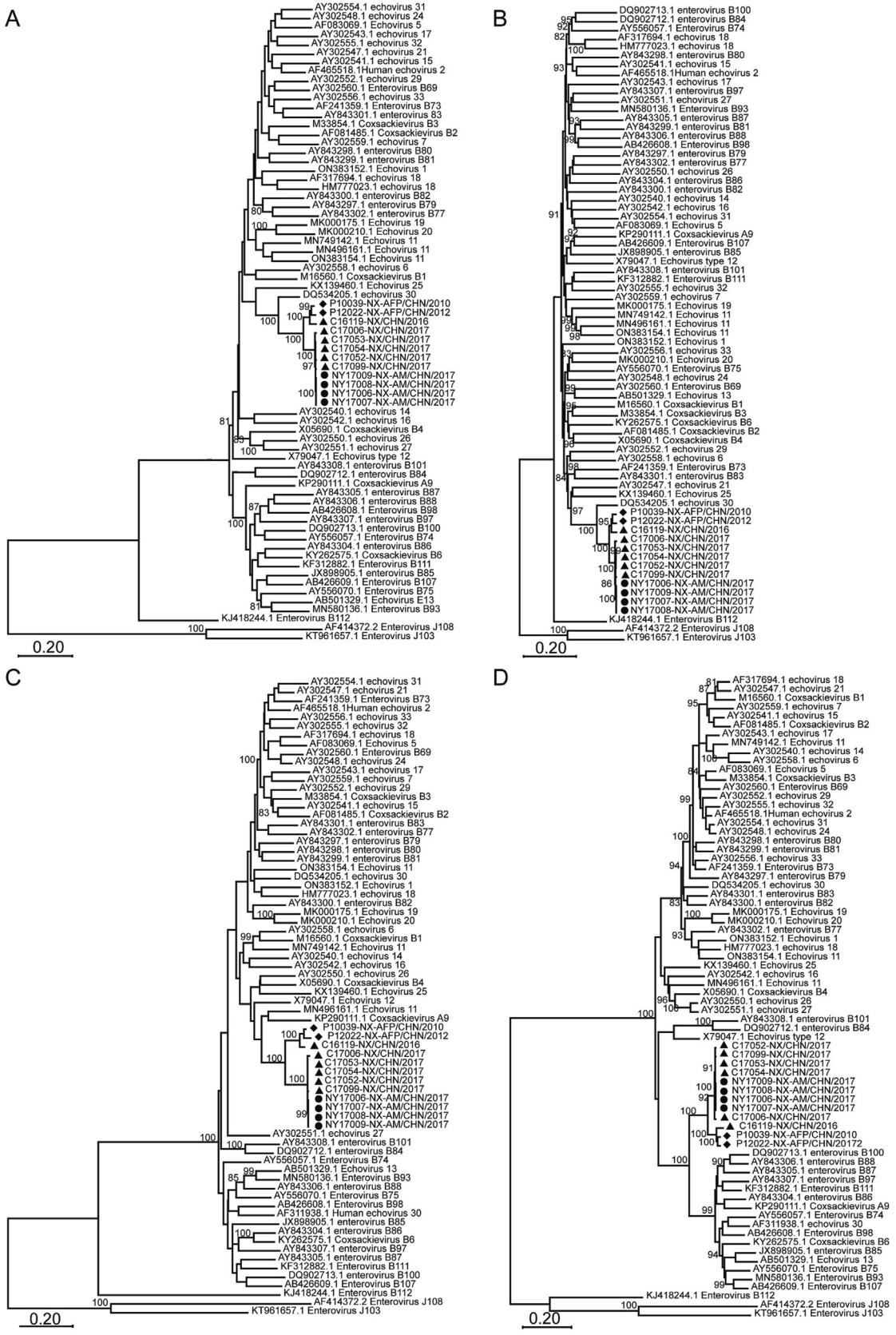

Fig. 2. Maximum-likelihood phylogenetic trees based on the *VP1* genes of echovirus 30 (E30) strains and the *P1*, *P2*, and *P3* coding regions of enterovirus species B (EV-BEV) strains. A) Phylogenetic tree based on the genes of echovirus 30 (E30) strains. B) Phylogenetic tree based on the *P1* coding regions of EV-B strains. C) Phylogenetic tree based on the P2 coding regions of EV-B strains. D) Phylogenetic tree based on the *P3* coding regions of EV-B strains. Bootstrap values above 70% are shown at the nodes. The scale bar represents nucleotide substitutions per site. Triangles represent strains isolated from healthy contacts, solid circles represent strains isolated from aseptic meningitis (AM) cases, and diamonds represent E30 isolated from acute flaccid paralysis (AFP) cases.

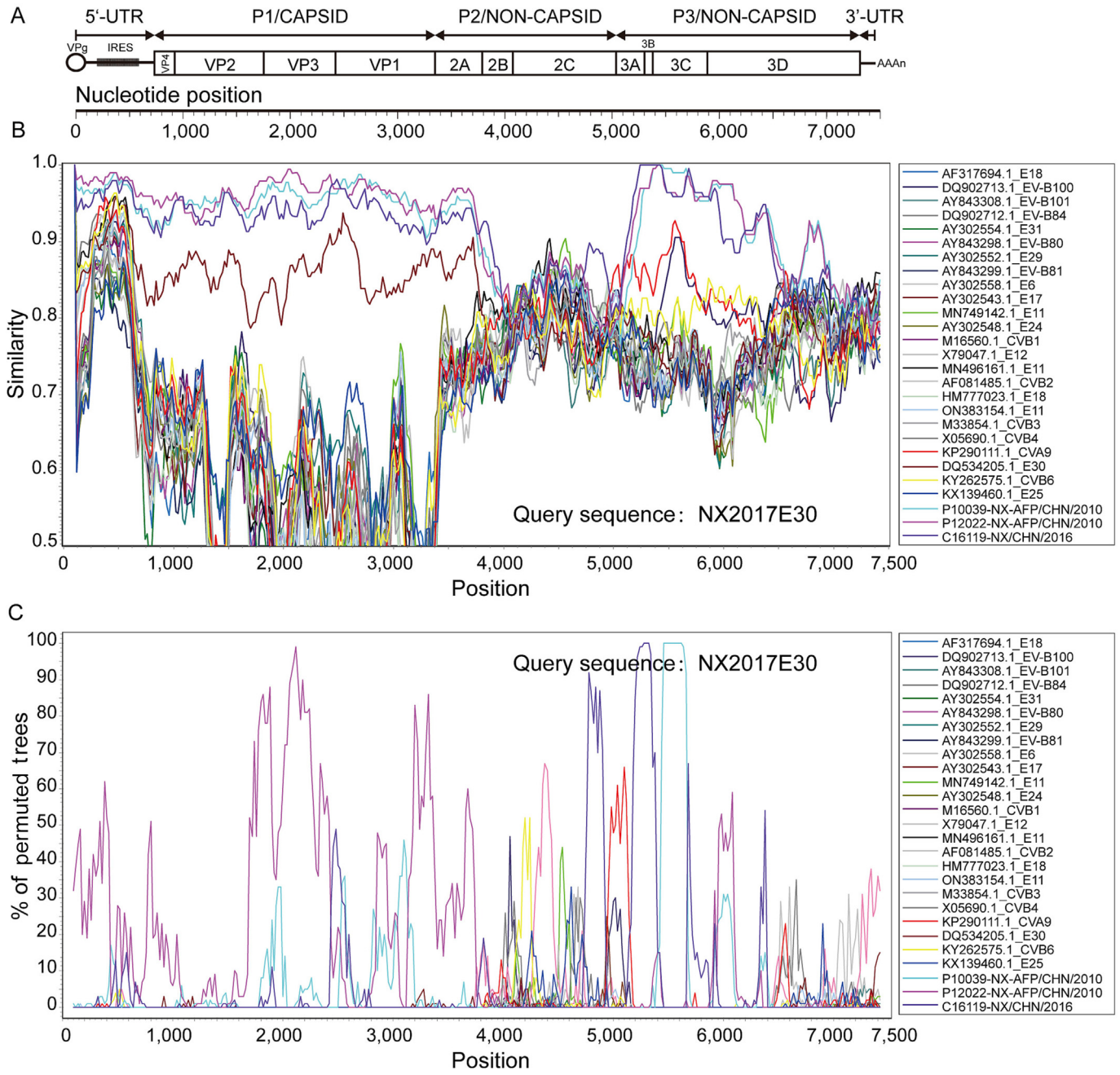

Fig. 3. Similarity plot analysis comparing strain Ningxia echovirus 30 (E30) with enterovirus B (EV-B) strains, based on the full-length genomes. The 2017 E30 strains were used independently as query sequences. The nucleotide sequence of each strain was compared with those of strains of EV-B, using Simplot software version 3.5.1, with a sliding window of 200 nucleotides moving in steps of 20. Colored lines indicate the strains described in this article, and other strains are indicated by gray lines. The whole genome Simplot BootScan analysis was conducted with the query sequence of NX2017E30 strain. A) The enterovirus genomic organization is shown at the top. B) Similarity plot analysis. C) Bootscanning analysis on the putative recombinant strain and its parental sequences.

clinical presentation. Genetic evolution analysis of the *VP1* region showed that the isolates from Ningxia in 2016 and Gansu in 2015 belonged to the same transmission chain from lineage H. The E30 prevalent in 2017 was on the same lineage as the 2016 E30 in Inner Mongolia, a new mutant strain different from the one prevalent in Gansu and Ningxia in 2017. Unlike in Inner Mongolia, the epidemic strain formed an independent transmission chain in Ningxia, suggesting a closer spread of E30 between neighboring provinces.

Interestingly, meningitis and asymptomatic infection populations in 2017 belonged to the same epidemic strain, but there were significant differences in age distribution that sampling requirements may have influenced. Surprisingly, many of the cases reported recent contact with children. However, in a New Zealand hospital in 2017, the outbreak was caused by an E30 strain closely related to strains reported from Ningxia, aged 16-55 years [20]. Therefore, we believe that further studies on the pathogenicity of E30 in different populations are needed.

Recombination plays an important role in the evolution of enteroviruses[21,22]. Studies have reported that new E30 lineages undergo recombination with other EV-B types before they emerge, that recom-

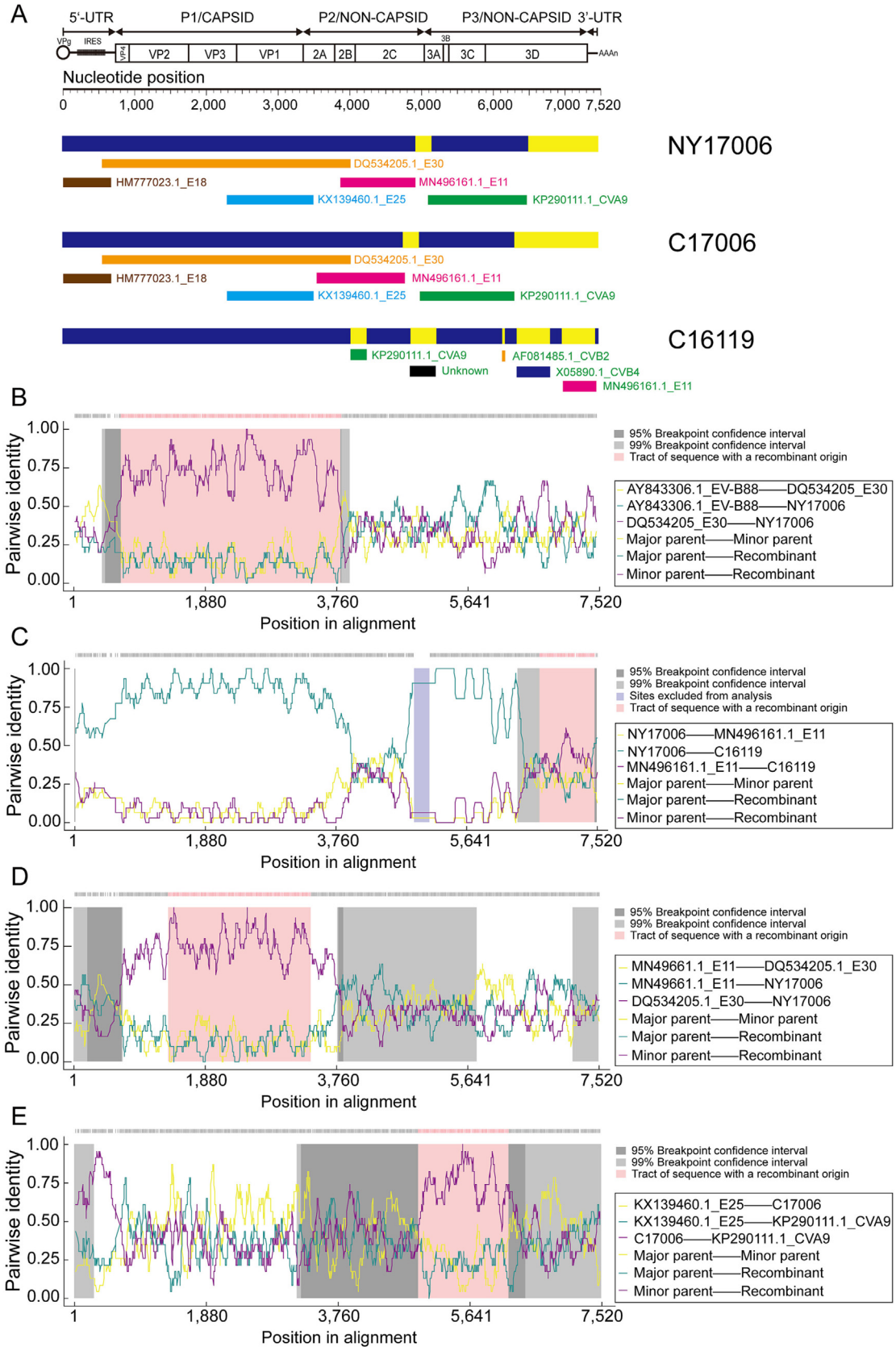

Fig. 4. Recombination analysis results using RDP4 software. A) A schematic sequence display depicting color-coded representations of the analyzed sequences and the locations of detected recombination events; Estimated recombination breakpoint positions echovirus 30 strain 2010, 2012, and 2016 with closely related strains. B) to E), the RDP4 analyses of putative recombinants. The plots illustrate the statistical evidence underlying the detection of individual user-selected recombination events.

bination generates new viral forms that are derived from co-circulating E30 and/or other EV-B types [23,24], and that the emergence of such new lineages plays an important role in large-scale outbreaks [25,26]. Research has also shown that recombination events in EVs are almost exclusively detected at the edges of the structural *P1* region or within the non-structural 5′ UTR, *P2*, or *P3* regions [27,28]. Generally, *P2* and *P3* are most enriched in recombination events [29,30]. Data analysis using RDP4 and SimPlot software indicated intra-typic and inter-typic recombination in the prevalent E30 in Ningxia, with recombination signals detected in the *P1*, *P2*, and *P3* regions. The recombinant genotypes were E30, E11, E6, CVB4, E25, CVA9, and E21. Notably, Nikolaidis [31] identified E30, E6, E25, CVB5, CVB4, and CVA9 as prominent recombination partners, with E25 emerging as one of the most recombined genotypes. Our results show recombination events between the strains in this study and other enteroviruses, such as the E18, E30, E11, E25, CVA9, and E21 genotypes. The recombination regions were consistent with the recently studied recombination hotspots of E30 in the 5′ UTR-VP4 junction region, *2A*, *2B*, and *2C* regions. Recombination events were also detected in the *P3* region, albeit at a lower frequency. This study showed no direct evidence of recombination of the 2017-prevalent E30 strain in Ningxia with E6 and E11, but recombination with E6 and E11 was detected in the pre-2017-prevalent sequences. However, Ninxia detected recombination in both the 2017-prevalent and pre-2017-prevalent E30 strains from Ningxia. We observed that intra-typic recombination was more frequent in the *P1* region, and inter-typic recombination occurred mainly in the *P2* and *P3* regions. Recombination sites in the *P2* region were more numerous and complex than those in regions *P1* and *P3*. Several national and international studies have confirmed the presence of many recombination events in E30, which appear to be relatively randomly distributed and lead to a complex sequence mosaic [32,33]. Recent studies on recombination mechanisms consider the EV genome a multistep combinatorial evolutionary process, constructing an assembly of genomic segments or recombination modules. The genome seems to generate a precursor non-homologous recombinant genome through an initial intergenomic recombination event, followed by the creation of multiple homologous recombinant genomes through one or more subsequent genomic rearrangements. Replication copy selection mechanisms and non-replication break-linkage mechanisms then lead to the further evolution of non-homologous recombinant genomes through homologous recombination with recombination sites in *2A* regions that undergo genome evolution through recombination module exchange [22]. In this study, it is possible that the E30 was the product of this recombination mechanism. Recombination can lead to increased viral pathogenicity and adaptability [34,35], which may be the main reason for the massive outbreak epidemic of this new spectrum.

The mechanisms underlying the complex circulation patterns of E30 and other enteroviruses and the impact of population immune changes, antigenic changes, viral diversification, pathogenicity, and recombination must be further explored. Through this study, we found the importance of continuous dynamic surveillance, which on the one hand, can be used to identify a large number of cryptically transmitted viruses in healthy populations while providing forewarning and early warning regarding outbreaks. However, to help identify emerging strains, it can help clarify the circulation and evolution of enteroviruses and gradually help to reveal their complex nature.

Ethics statement

This study did not involve human experimentation. All fecal specimens were obtained from the Ningxia disease surveillance system and written informed consent was obtained. All sample collection and experimental protocols were approved by the Ethics Review Committee of the Ningxia Hui Autonomous Region Disease Control and Prevention and operated according to approved guidelines.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Fang Yuan: Writing – original draft, Data curation. **Xinfeng Wei:** Resources. **Xueping Ma:** Project administration, Supervision. **Jiangtao Ma:** Data curation. **Xuemin Ma:** Data curation. **Xiaoqiang Sun:** Investigation. **Min Cao:** Investigation. **Juan Zhou:** Data curation. **Wei Zhang:** Investigation. **Hui Chen:** Investigation. **Rui Wang:** Data curation. **Jichen Li:** Data curation. **Qiang Sun:** Conceptualization, Writing – review & editing.

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