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BRIEF REPORT

An outbreak of encephalitis associated with echovirus 19 in Uttar Pradesh, India, in 2011

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Abstract A sequence-independent single-primer amplification method and a modified enterovirus VP1 gene typing primer were used for identification of echovirus 19 and enterovirus 101, which remained undiagnosed by standard enterovirus molecular typing methods. Six different serotypes were identified during this study, with the predominance of ECV 19 (n = 20) followed by echovirus 21 (n = 3), EV 69 and EV 101 (n = 2 each), coxsackievirus B5 and ECV 27 (n = 1 each). To our knowledge, this is the first report of enteroviruses 69 and 101 in encephalitis cases in India.

Abbreviations

EV	Enterovirus
ECV	Echovirus
CV	Coxsackie virus
CSF	Cerebrospinal fluid
RT-PCR	Reverse transcriptase polymerase chain reaction
NCR	Non-coding region
RD	Human rhabdomyosarcoma cells
HEp 2	Human epithelium larynx
CPE	Cytopathic effect
SISPA	Sequence independent single primer amplification

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Enteroviruses (EVs) belong to the genus *Enterovirus* in family *Picornaviridae*. They are small, non-enveloped, positive-strand RNA viruses comprising more than 110 serotypes divided into four species: *Enterovirus A-D* [1]. The clinical manifestations of EVs range from asymptomatic infection to more-severe diseases, including myocarditis, acute flaccid paralysis, meningitis and encephalitis [2]. Coxsackievirus (CV) A9, A10, and B5, echovirus (ECV) 4, 5, 9, 11, 19, and 30 and EV 71, 75, 76 and 89 have been reported in sporadic encephalitis cases or in epidemic form in different parts of the world, including India [3–8].

The conventional method for EV serotype identification is the isolation of the virus in cell culture, followed by neutralization with mixed hyperimmune equine serum pools and specific monovalent polyclonal antisera. However, due to the problem of low sensitivity, untypeable strains, and the emergence of antigenic variants, this method has been replaced by molecular methods that are based on RT-PCR and partial sequencing. Currently, molecular diagnosis of EVs is based on the amplification of the highly conserved 5′ untranslated region (5′UTR) for EV detection, followed by amplification and sequencing of part of the VP1 capsid region for serotype identification and phylogenetic analysis [9–11].

In Uttar Pradesh state of India, encephalitis is a significant cause of morbidity and mortality in children each

year. Japanese encephalitis virus (JEV) was the main etiologic agent in encephalitis cases in this state since the first major epidemic in 1978 [12]. However, due to JEV vaccination in this state through government vaccination programs, the incidence rate of JEV has been declining from 2.3 in 2010 to 0.58 in 2012 per 100,000, people but the number of encephalitis cases is still increasing [13]. EVs have been reported in encephalitis cases in endemic or epidemic form this area [7, 8, 14]. Currently, there is no vaccine available for EVs; continuous molecular epidemiological study is necessary to detect new serotypes or variants and establish epidemiological links between cases during outbreaks [7–9, 14, 15].

One hundred twenty-eight children with a clinical diagnosis of encephalitis were prospectively investigated for viral agents between March 2011 and February 2012. Cerebrospinal fluid (CSF) specimens were collected from children with clinical symptoms of encephalitis admitted to King George's Medical University and Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, Uttar Pradesh. A case of encephalitis was defined as fever with altered sensorium lasting more than 24 h with seizures and/or CSF pleocytosis with neuroimaging findings indicating parenchymal involvement. Written informed consent was taken from a patient's parents or legal guardians. This study was approved by the Institutional Ethical Board (ref. no. A-33:PGI/SRF/IEC/53/18.02.2011).

Viral nucleic acid was extracted from CSF samples using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Detection of EV, JEV, herpes simplex virus (HSV) 1 and 2, cytomegalovirus, West Nile virus, measles virus, dengue virus and chikungunya virus was performed using Geno-Sen's Rotor Gene Quantitative Real-Time PCR Kit (Genome Diagnostics, India) specific for each virus in a Rotor-Gene 6000 real-time instrument (Corbett Research, Victoria, Australia) according to the manufacturer's protocol. Immunoglobulin M capture ELISA was also used to detect JEV and HSV antibodies in CSF samples.

Molecular typing of EVs was performed by semi-nested reverse transcription polymerase chain reaction (RT-PCR) amplification of the VP1 region of the virus genome using the primers AN88 and AN89 according to Nix et al. [10]. The amplicons were separated by 2 % agarose gel electrophoresis, purified using a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), and sequenced on an ABI PRISM automated sequencer by Sequencher Tech, India, with the same primers (AN88 and AN89). GenBank basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/Blast) was used for serotype identification.

CSF samples that remained negative for a viral etiologic agent or could not be typed by EV VP1 gene PCR were inoculated onto human rhabdomyosarcoma cells (RD) and human epithelium larynx (HEp 2) cells. Virus isolates producing a reproducible cytopathic effect on RD cells were processed for virus identification using a sequence-independent single primer amplification (SISPA) method as described previously [16]. Twenty to 30 recombinant plasmids for each sample were sequenced on an ABI PRISM automated sequencer by Macrogen, Korea, with the primers PJET 1.2 F and PJET 1.2 R. Sequences were analyzed for similarities to known viruses, using the tBLASTx program.

We designed a new primer, AN 89, due to a mutation in the primer-binding site and screened all original clinical specimens for EVs using the modified AN89 and AN 88 primers. In brief, EV VP1 gene first PCR was done using an AffinityScript One-Step RT-PCR Kit (Agilent Technologies) in a 50-μl reaction mixture containing 5 μl of RNA, 25 μl Herculase II RT-PCR 2 × Master Mix, 0.5 μl of AffinityScript RT/RNase Block, 0.5 μl containing 50 pmol each of modified AN89 (CCAGCMCTSA-CAGCAGYNGARAYNGG) and AN88 (TACTGGAC-CACCTGGNGGNAYRWACAT), and 31.5 μl of RNase-free water. Amplification was performed on an Applied Biosystems GeneAmp PCR System 9700 (Thermo Fisher Scientific) with a single cycle of 50 °C for 30 minutes and 95 °C for 2 minutes, followed by 45 amplification cycles of denaturation for 30 seconds, primer annealing at 58 °C for 30 seconds, extension at 72 °C for 45 seconds, and a final extension step at 72 °C for 5 minutes. The specific product was sequenced on an ABI PRISM automated sequencer by Macrogen, Korea, with both forward and reverse primers.

Multiple sequence alignments with the respective reference strain sequences were generated using the Clustal omega program (http://www.ebi.ac.uk/clustalw). The phylogenetic tree was inferred using the Kimura 2-parameter algorithm and the neighbor-joining algorithm with 1,000 bootstrap replicates in MEGA 6.0 software (http://www.megasoftware.net) [17]. Sequences obtained from EV isolates in this study have been submitted to the GenBank database under accession numbers KT716273-KT716301.

One hundred twenty-eight children with suspected viral encephalitis were enrolled between March 2011 and February 2012. Of 128 CSF samples, 29 (22.6 %) were positive for EV, 14 (10.9 %) for JEV, 11 (8.6 %) for HSV, 4 (3.4 %) for dengue virus and 2 (1.6 %) for measles virus. EV RNA was detected in 27 (21.1 %) of 128 CSF samples by real-time RT-PCR and 7 (5.5 %) by VP1 PCR (Table 1). The demographic and dominant clinical features of encephalitis patients in this study were age 4.8 ± 1.4 (mean ± SD), male (55.47 %), female (44.53 %), high-grade fever (100 %), altered sensorium (88.28 %), convulsions (80.47 %), headache (79.69 %), and personality change (48.44 %).

Due to the increased rate of PCR failure in the EV VP1 gene, we attempted virus isolation on RD and HEp 2 cells

Table 1 Enterovirus serotypes identified in the present study using the modified AN 89 primer

Enterovirus type	Enterovirus 5′UTR real-time PCR	Enterovirus VP1 gene PCR	Enterovirus VP1 gene PCR with modified AN 89 primer
Coxsackievirus B5	1	1	1
Echovirus 19	20	0	20
Echovirus 21	3	3	3
Enterovirus 27	1	1	1
Enterovirus 69	2	2	2
Enterovirus 101	0	0	2

in 70 CSF samples that were negative for a viral etiologic agent or were positive for EV by real-time PCR but could not be typed by EV VP1 gene PCR. Out of 70 CSF samples, four were produced a reproducible cytopathic effect on RD cells. ECV 19 was identified in all isolates by using tBLASTx analysis of sequences generated by the SISPA method. Retrospective screening of all original clinical specimens by using a new modified AN 89 primer; ECV 19 (n = 20) and HEV 101 (n = 2) were identified, which were previously negative for EV when using a standard EV molecular typing method based on real-time PCR and a VP1 typing method. A total of six different serotypes were identified during this study, with the predominance of ECV 19 (n = 20) followed by ECV 21 (n = 3), EV 69 and EV 101 (n = 2 each), coxsackievirus B5 and ECV 27 (n = 1 each).

All EV strains from this study met the serotype identification criteria for homologous serotypes, including at least 75 % nucleotide or 88 % amino acid sequence identity in the VP1 region [11]. The nucleotide sequence divergence range for each serotype (CV B5, ECV 19, ECV 21 and 27, EV 69 and EV 101) from the respective reference serotype was 20.08 %, 21.2-24.9 %, 18.37-21.22 %, 22.54 %, 21.9-22.31 %, and 15.29-14.05 % respectively.

A salient feature of the present study was the failure of EV molecular typing methods commonly used in our laboratory for the past seven years to detect and type ECV 19 (EV 5′UTR positive/VP1 negative) and HEV 101 (EV 5′UTR negative/VP1 negative). Phylogenetic analysis of ECV 19 sequences from the present study with all available sequences shows the emergence of a new genotype that is more closely related to Indian strains isolated from encephalitis patients during 2008-2009. All ECV 19 strains isolated from encephalitis patients formed a separate cluster from other ECV 19 strains isolated from gastroenteritis, acute flaccid paralysis, and environmental specimens from India and other parts of the world (Fig. 1). These findings suggest that a new genotype of ECV 19
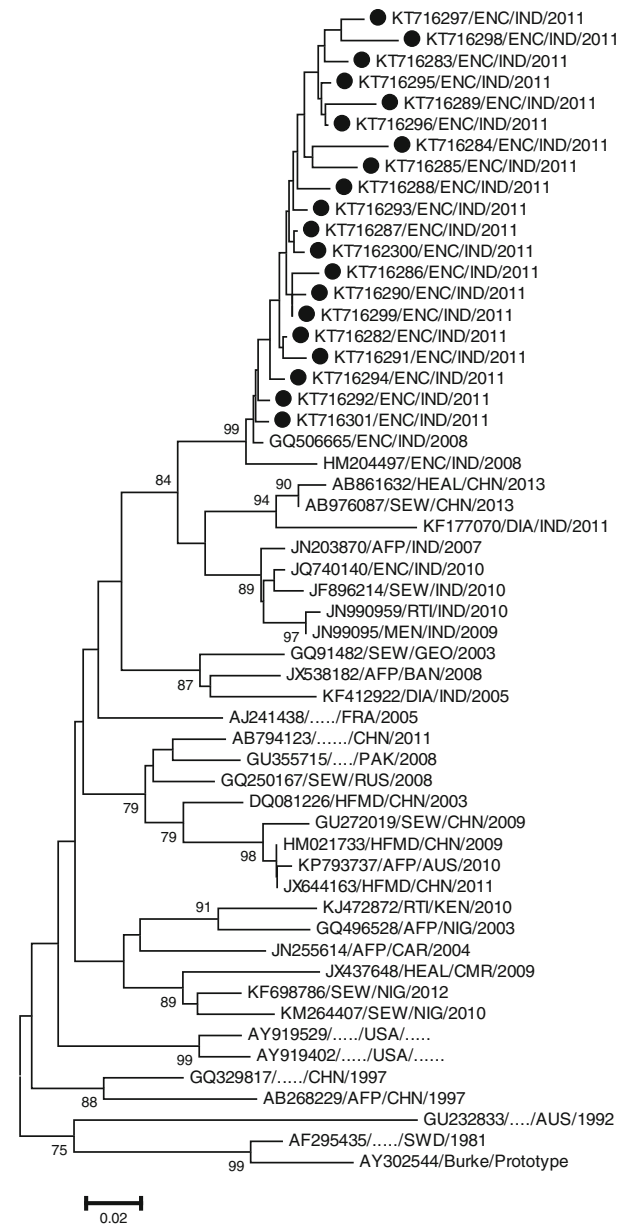
Fig. 1 Phylogenetic analysis of a partial nucleotide sequence of the region encoding VP1 of echovirus 19 strain from this study (indicated by a filled circle) and other worldwide reference strains. The evolutionary history was inferred by the neighbor-joining method, using the MEGA 6 program [17]. The scale bar indicates nucleotide substitutions per site. Bootstrap values in 1,000 pseudoreplicates > 70 are indicated at the branch nodes. Each strain is represented as accession no./clinical manifestations/country/year of isolation, if available, using the web data from GenBank. A dotted line indicates that there is no information about the year of isolation. Echovirus 19 sequences identified during this study are indicated by filled round circles. ENC, encephalitis; HEAL, healthy; AFP, acute flaccid paralysis; DIA, diarrhoea; RTI, respiratory tract infection; MEN, meningitis; HFMD, hand, foot and mouth disease; SEW, sewage; IND, India; CHN, China; GEO, Georgia; FRA, France; PAK, Pakistan; RUS, Russia; AUS, Australia; KEN, Kenya; CAR, Central African Republic; CMR, Cameroon; NIG, Nigeria; USA, United States of America; SWE, Sweden; NGR, Nigeria

with increased pathogenicity has evolved in the studied area.

HEV 101 is a newly identified serotype. Only two sequences of HEV 101 are available in the NCBI database. The difficulties in the diagnosis of EV 101 by standard methods highlight the need for improved molecular typing methods for enterovirus detection and typing [18–20]. This is the first report of HEV 69 and 101 in encephalitis cases in India. However, further study based on the complete genome sequence and serological surveillance is necessary for better understanding of their evolution and pathogenesis.

Compliance with ethical standards

Conflict of interest   None.

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