*Original Article*

# High frequency of enterovirus serotype circulation in a densely populated area of India

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**Abstract**

Introduction: In the state of Uttar Pradesh in India, enteroviruses are a significant cause of infection presenting in endemic or epidemic forms. The present study aimed to use molecular methods to identify enterovirus serotypes in clinical specimens to determine their circulation in the community.

Methodology; A total of 320 clinical specimens were collected between January 2009 and December 2010 from children younger than 15 year of age in northern India. Reverse- transcription (RT) real time PCR and semi-nested RT PCR targeting the 5′untranslated region and VP1 region was used for the detection and identification of enterovirus serotypes.

Results: The enterovirus genome was detected in 79 (24.7%) of 320 clinical specimens by real time PCR. Central nervous system syndrome (CNS) was the most common clinical manifestation (n=32, 62.74%), followed by respiratory tract infection (n=8, 15.69%), acute febrile illness (n=7, 13.73%), and gastrointestinal disease (n=4, 7.84%). A total of 32 different serotypes were identified with the predominance of coxsackievirus B5 and echovirus 6. Phylogenetic analysis of partial VP1 gene sequences from this study showed that many enterovirus serotypes showed good similarity with strains from America and Europe in comparison to neighbouring Asian countries.

Conclusions: To our knowledge this is the first study of enterovirus prevalence from northern India based on unbiased molecular methods which leads to the identification of fifteen different enterovirus serotypes. The high frequency of enterovirus B species serotypes circulation may be an important cause of CNS infection in the children of this region.

**Key words:** molecular identification; VP1; human enterovirus B; phylogenetic analysis

*J Infect Dev Ctries* 2013; 7(6):475-483.. doi:10.3855/jidc.2413

(Received 17 November 2011 – Accepted 4 January 2012)



## Introduction

Human enteroviruses (HEVs) belonging to the genus Enterovirus in family Picornaviridae are divided into four species on the basis of molecular and biological properties: HEV A (22 serotypes), HEV B (60 serotypes), HEV C (20 serotypes), and HEV D (4 serotypes) [1]. HEVs infect millions of people worldwide causing asymptomatic infection to a comprehensive range of clinical diseases such as hand-foot-and-mouth disease, aseptic meningitis, encephalitis, acute flaccid paralysis, myocarditis and neonatal sepsis-like diseases [2].

Although HEVs are ubiquitous, some serotypes that may be endemic in a particular geographical area might emerge into new variants, periodically causing epidemics and transmission to a different geographical area [3-5]. Continuous molecular epidemiological study of HEV serotypes is necessary to study the changing patterns of enterovirus infection and disease association, as well as to detect new serotypes or variants and establish epidemiological links among cases during outbreaks [2,6 -7].

The conventional method for typing of HEV serotypes is isolation of the virus in cell culture, followed by neutralization with mixed hyperimmune equine serum pools and specific monovalent polyclonal antisera. The problems with this method are that i) some HEV serotypes do not grow in culture,and ii) that the antigenic variants emerge quite often [5,7,10]. In recent years, these methods have been replaced by molecular methods based on reverse transcription-PCR and sequencing. Currently molecular diagnosis of HEVs is based on the amplification of the highly conserved 5′

untranslatedregion (5′ UTR) for enterovirus detection followed by amplification and sequencing of the partial VP1 capsid region for typing [8-11].

Uttar Pradesh state in northern India holds one fifth of the population of the country, with the lowest human development indices. Approximately 50% of the population is children younger than 15 year of age who mostly live in rural areas. HEVs are a significant cause of infection in the children of this region, presenting in endemic or epidemic form [12-16]. Previous reports about HEV circulation have been published from this region [12-16]. Both conventional [12] and molecular methods [13-14] were used in these studies. The 5′UTR region was targeted for detection of these viruses in cases of acute flaccid paralysis [13-14], and the VP1 region was targeted for detection of the virus in cases involving acute encephalitis syndrome cases [15-16]. Unfortunately the sequence of the 5′ UTR region provides no information about the serotype of the infecting virus [17],and serotype identification during an epidemic or outbreak does not provide the actual prevalence of enterovirus serotype circulating in the community. We therefore conducted this study using the molecular method of enterovirus detection and identification in clinical specimens from both patients with suspected enterovirus infection and healthy children over a two-year period to determine the occurrence and clinical manifestations of the virus in this region.

## Methodology

### *Patients and clinical samples*

A total of 320 children (aged ≤ 15 years) were enrolled between January 2009 and December 2010 from Uttar Pradesh, India. A total of 160 clinical specimens (cerebrospinal fluid [n = 25], throat swab [n = 60], serum [n = 15], and stool [n = 60]) were collected from patients who had received medical care at General Hospital, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, Uttar Pradesh, or whose clinical specimens were sent for enterovirus diagnosis at the Department of Microbiology, SGPGIMS Lucknow, Uttar Pradesh. An equal number of samples were collected from 160 healthy children who were living in the same locality as the infected children. All specimens were immediately frozen and transported to the laboratory and stored at –70°C until further use. The study protocol was approved by the Institutional Ethical Committee (A-10:PGI/EP/EC/44/28.11.2008). Written informed consent was obtained from the parentsorguardians of all patients and healthy controls who participated in this study.

### *RNA extraction and detection of HEV by real time PCR*

Stool samples were processed with chloroform and centrifuged according to the World Health Organization (WHO) Polio Laboratory Manual, 2004 [18]. Throat swabs were dissolved in viral transport media (HiMedia, Mumbai, India) and centrifuged at 4,000 × g for 20 minutes. Cerebrospinal fluid and serum were used for RNA extraction. Viral RNA was extracted from 125 µl of centrifuged stool supernatant/throat swab supernatant/cerebrospinal fluid/serum with a QIAamp Viral RNA mini kit (QIAGEN, Inc., Valencia, CA, USA), using a protocol according to the manufacturer's instructions. The extracted RNA was kept at –70°C until use. HEV nucleic acid was detected by using Geno-Sen'sEnterovirus Rotor Gene Quantitative Real Time PCRKit (Genome Diagnostics Pvt Ltd, New Delhi, India) specific for the 5′ UTR of EVs in a Rotor-Gene 6000 Real-Time PCR Detection System (Corbett Research, Mortlake, Victoria, Australia) according to the manufacturer's protocol. A standard curve was generated using positive controls with known viral copies provided in the kit.

### *Molecular typing of HEV*

HEV serotypes were determined in all real time PCR positive samples by semi-nested RT-PCR and sequencing of a partial VP1 region according to Nix *et al*. [9] with some modifications. In brief,cDNA synthesis was performed in a 10 µl reaction mixture containing 5 µl of RNA, 10 mM each deoxynucleoside triphosphate (dNTP), 2 µl of 5x reaction buffer (Invitrogen), 0.01 M dithiothreitol (Invitrogen), 1 µl of random hexamers primer, 20 U of RNasin (Roche Applied Science), and 100 U of SuperScript III reverse transcriptase (Invitrogen). The reaction mixture was incubated at 22°C for 10 minutes, 42°C for 45 minutes, and 95°C for 5 minutes. The first PCR run (final volume 25 µl) consisting of 2 µl cDNA, 2.5 µl of 10X PCR buffer (Roche Applied Science), 10 mM each dNTP, 50 pmol each of primers 224 and 222, and 1.25 U of Taq DNA polymerase (Roche Applied Science) was subjected to 40 cycles of amplification (95°C for 30 seconds, 45°C for 30 seconds, 60°C for 45 seconds). One microliter of the first PCR product was added to a second PCR run (final volume 50 µl) consisting of 10 mM each dNTP, 40 pmol each of primers AN89 and AN88, 5 µl of 10X PCR buffer II

(Applied Biosystems), and 2 U of Taq DNA polymerase (Applied Biosystems). The PCR was performed at 94°C for 10 minutes prior to 35 amplification cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 25 seconds with a final extension at 72°C for 10 minutes. Second round PCR products were treated with ExoSap-IT (USB) before direct sequencing. Purified DNA templates were sent to Vimta Company, India, for sequencing by using both primers AN 89 and AN 88 on an automated sequencer (Applied Biosystems, Foster City, CA).

### *Phylogenetic analysis*

Molecular identification of each isolate was performed by pairwise comparison of the VP1 amplicon sequence with a database of all EV serotypes using the BLAST program (www.ncbi.nlm.gov/BLAST) from GenBank. Strains that had the highest nucleotide similarity (at least 75% identical in VP1 sequence) were interpreted as belonging to the same serotype [8]. Multiple sequence alignment was performed using the Clustal W program in MEGA 4 software (www.megasoftware.net). A phylogenetic tree was computed using the Kimura 2-parameter and the neighbor-joining method with bootstrap 1,000 replicates in MEGA 4 software [19].

### *Data analysis*

Statistical analyses were performed using SPSS version 16 (IBM, SPSS Inc, Chicago, USA). The prevalence for each species in healthy and diseased children was computed with 95% confidence intervals. A P value of less than 0.05 was considered to be statistically significant.

### *Nucleotide sequence accession numbers*

The sequences reported in this study have been deposited in the nucleotide sequence database under accession numbers (HQ664088-HQ664116, JN990931-JN990979). The sequence of coxsackievirus A4 was less than 200 bp and was not submitted to GenBank.

## Results

### *Prevalence and epidemiology of HEV infections*

The HEV genome was detected in 79 (24.7%; 95% CI, 20 - 29.4%) of 320 clinical specimens from 320 children. A statistically significant (P= 0.002) relation was found between the rate of HEV detection in healthy children (17.5%) and children with disease (31.9%). The viral copy number was higher in individuals with disease in comparison to that in healthy children (P=0.003) (Table). Central nervous system syndrome (CNS) was the most common clinical manifestation (n = 32, 62.74%) found in the diseased children, followed by respiratory tract infection (n = 8, 15.69%), acute febrile illness (n = 7, 13.73%), and gastrointestinal disease (n = 4, 7.84%).

Among the 79 children positive for HEV, 47 (59.49%) were younger than three years of age. A pattern of low rate of HEV detection rate with increase of age (4 years to 6 years [22.78%], 7 years to 9 years [6.33%], 13 years to 15 years [2.53%]) was observed; however, HEV detection rate was high in the 10 years to 12 years age group (8.86%) (Table). There was no significant association between enterovirus detection and gender in healthy and diseased children. A seasonal pattern was observed throughout the study with a high detection rate from August to September (rainy season; 20.5 %, 95% CI, 10.2% to 30.8%), to lower rates in April to May (summer season; 11.4%, 95% CI, 5.1- 19.0%) and December to January (winter season; 9%, 95% CI, 2.5% - 15.2%) (Figure 1).

The HEV serotype was identified in all 79 (100 %) real time PCR positive samples. A total of 32 different serotypes were detected (Table). Coxsackie virus (CV) B5 and echovirus (ECV) 6 were the most dominant serotypes (11.4% each) followed by CV B3 (6.3%), CV A13, CV B6, ECV 3, ECV 20 and ECV 29 (5.1% each), ECV 11, ECV 24 and enterovirus 75 (3.8% each). HEV B was the most prevalent species in healthy and diseased children (84.8%; 95% CI, 75.9% to 92.4%); there were no significant differences between HEV species in healthy children (85.7%) and children with disease (84.3%). HEV A species was present only in children with disease (6.3%; 95% CI, 1.3% to 12.7%). The prevalence of HEV C species in healthy and diseased children was 8.9% (95% CI, 2.5% to 15.2%) with a high detection rate in healthy children (14.3%) in comparison to children with disease (5.9%). No enterovirus from HEV-D group was detected.

To our knowledge, this is the first report of enterovirus serotypes (*i.e.*, CV A4, CV A8, CV A13, CV A17, CV A21 and ECV 1, 2, 20, 24, 27, 29, 31, 33 and EV 74, EV 80 from India. CV A13, CV A17, ECV 1, 2 and ECV 31) detected from asymptomatic children. In contrast, CV A4, CV A8, CV A21, ECV 20, 24, 29, 33, EV 74 and EV 80 were associated with different clinical manifestations including respiratory tract infection, acute febrile illness, and encephalitis (Table).

**Table.** Demographic and clinical presentation of enterovirus serotypes detected in the present study

| No | Month of collection | Clinical Presentation | Age in year | Clinical specimen | EV Serotype | Virus Log copies |
|---|---|---|---|---|---|---|
| **1** | 01/2009 | RTI | 0.2 y | Throat swab | CV A4 | 4.0 |
| **2** | 01/2009 | AFI | 1.4 y | Stool | CV A8 | 4.2 |
| **3** | 01/2010 | RTI | 2.8 y | Throat swab | CV A10 | 3.2 |
| **4** | 02/2010 | GI | 2.4 y | Stool | CV A10 | 2.8 |
| **5** | 01/2010 | CNS | 2.4 y | Throat swab | EV 76 | 2.4 |
| **6** | 03/2009 | Healthy | 2.4 y | Stool | *CV B1 | 3.0 |
| **7** | 03/2009 | AFI | 0.9 y | Stool | *CV B1 | 4.9 |
| **8** | 04/2009 | Healthy | 2.9 y | Stool | CV B2 | 3.7 |
| **9** | 07/2009 | Healthy | 7.6 y | Stool | *CV B3 | 3.2 |
| **10** | 09/2009 | Healthy | 2.9 y | Stool | *CV B3 | 3.0 |
| **11** | 09/2009 | Healthy | 2.8 y | Stool | *CV B3 | 3.7 |
| **12** | 05/2010 | CNS | 1.4 y | Throat swab | *CV B3 | 4.8 |
| **13** | 05/2010 | CNS | 11.8 y | CSF | *CV B3 | 4.4 |
| **14** | 04/2009 | Healthy | 4.4 y | Stool | *CV B5 | 3.3 |
| **15** | 05/2009 | Healthy | 2.0 y | Stool | *CV B5 | 4.9 |
| **16** | 09/2009 | Healthy | 2.1 y | Stool | *CV B5 | 3.7 |
| **17** | 09/2009 | CNS | 7.7 y | Throat swab | *CV B5 | 4.8 |
| **18** | 05/2010 | CNS | 8.4 y | Stool | *CV B5 | 4.7 |
| **19** | 05/2010 | CNS | 4.1 y | Stool | *CV B5 | 4.1 |
| **20** | 06/2010 | CNS | 5.4 y | Stool | *CV B5 | 4.6 |
| **21** | 07/2010 | RTI | 2.0 y | Throat swab | *CV B5 | 4.2 |
| **22** | 08/2010 | AFI | 2.9 y | Stool | *CV B5 | 4.8 |
| **23** | 05/2009 | Healthy | 2.1 y | Stool | *CV B6 | 3.2 |
| **24** | 05/2009 | Healthy | 1.4 y | Stool | *CV B6 | 4.9 |
| **25** | 11/2010 | GI | 1.9 y | Stool | *CV B6 | 4.2 |
| **26** | 11/2010 | AFI | 1.2 y | Stool | *CV B6 | 5.4 |
| **27** | 07/2009 | Healthy | 5.4 y | Stool | ECV 1 | 4.2 |
| **28** | 03/2010 | Healthy | 3.0 y | Stool | ECV 2 | 4.2 |
| **29** | 03/2010 | CNS | 3.0 y | Throat swab | ECV 3 | 3.8 |
| **30** | 04/2010 | CNS | 2.6 y | Throat swab | ECV 3 | 4.9 |
| **31** | 09/2010 | CNS | 2.3 y | CSF | ECV 3 | 5.2 |
| **32** | 09/2010 | CNS | 2.7 y | Stool | ECV 3 | 5.4 |
| **33** | 05/2009 | Healthy | 2.0 y | Stool | *ECV 6 | 3.2 |
| **34** | 05/2009 | Healthy | 10.4 y | Stool | *ECV 6 | 4.0 |
| **35** | 07/2009 | Healthy | 3.4 y | Stool | *ECV 6 | 4.5 |
| **36** | 07/2009 | CNS | 4.0 y | Throat swab | *ECV 6 | 4.9 |
| **37** | 09/2010 | CNS | 4.1 y | Stool | *ECV 6 | 4.8 |
| **38** | 09/2010 | CNS | 10.6 y | Stool | *ECV 6 | 5.2 |
| **39** | 09/2010 | CNS | 2.0 y | Stool | *ECV 6 | 6.2 |
| **40** | 09/2010 | RTI | 8.4 y | Throat swab | *ECV 6 | 5.2 |
| **41** | 11/2010 | AFI | 4.7 y | Stool | *ECV 6 | 5.0 |
| **42** | 07/2009 | Healthy | 5.0 y | Stool | *ECV 11 | 4.2 |
| **43** | 09/2010 | CNS | 10.1 y | Stool | *ECV 11 | 5.1 |
| **44** | 09/2010 | CNS | 11.4 y | Stool | *ECV 11 | 5.6 |
| **45** | 07/2010 | Healthy | 1.9 y | Stool | ECV 12 | 4.2 |
| **46** | 05/2009 | Healthy | 1.8 y | Stool | *ECV 13 | 4.4 |
| **47** | 07/2010 | CNS | 2.1 y | Throat swab | *ECV 13 | 6.4 |
| **48** | 09/2009 | CNS | 2.2 y | Stool | ECV 14 | 4.0 |
| **49** | 07/2009 | CNS | 2.9 y | Throat swab | ECV 19 | 4.0 |
| **50** | 09/2010 | CNS | 2.0 y | Throat swab | ECV 19 | 5.2 |
| **51** | 07/2010 | Healthy | 2.1 y | Stool | *ECV 20 | 5.0 |
| **52** | 07/2010 | Healthy | 2.6 y | Stool | *ECV 20 | 4.2 |
| **53** | 07/2010 | CNS | 5.0 y | Stool | *ECV 20 | 6.2 |
| **54** | 07/2010 | AFI | 5.2 y | Stool | *ECV 20 | 5.8 |
| **55** | 11/2009 | CNS | 2.9 y | Stool | ECV 24 | 4.1 |
| **56** | 07/2010 | AFI | 14.2 y | Stool | ECV 24 | 5.2 |
| **57** | 07/2010 | AFI | 13.4 y | Stool | ECV 24 | 3.2 |
| **58** | 05/2010 | Healthy | 2.8 y | Stool | ECV 27 | 3.0 |
| **59** | 05/2010 | Healthy | 2.0 y | Stool | ECV 27 | 3.9 |
| **60** | 07/2009 | Healthy | 4.4 y | Stool | *ECV 29 | 4.2 |

**Table *(cont'd)*.** Demographic and clinical presentation of enterovirus serotypes detected in the present study

| | | | | | | |
|---|---|---|---|---|---|---|
| **61** | 07/2009 | CNS | 2.4 y | Throat swab | *ECV 29 | 5.2 |
| **62** | 07/2010 | CNS | 11.7 y | Throat swab | *ECV 29 | 4.0 |
| **63** | 07/2010 | RTI | 8.2 y | Throat swab | *ECV 29 | 5.8 |
| **64** | 04/2009 | GI | 1.9 y | Stool | ECV 30 | 5.1 |
| **65** | 05/2009 | Healthy | 2.4 y | Stool | ECV 31 | 4.6 |
| **66** | 11/2009 | CNS | 4.9 y | Stool | ECV 33 | 4.4 |
| **67** | 11/2009 | RTI | 2.0 y | Throat swab | ECV 33 | 4.7 |
| **68** | 09/2009 | CNS | 5.8 y | CSF | EV 74 | 4.8 |
| **69** | 07/2009 | CNS | 4.1 y | Stool | EV 75 | 4.3 |
| **70** | 05/2010 | CNS | 5.4 y | Throat swab | EV 75 | 2.2 |
| **71** | 05/2010 | CNS | 4.0 y | Throat swab | EV 75 | 2.8 |
| **72** | 04/2009 | CNS | 10.2 y | CSF | EV 80 | 3.9 |
| **73** | 07/2009 | Healthy | 1.9 y | Stool | *CV A13 | 3.8 |
| **74** | 07/2010 | RTI | 5.7 y | Throat swab | *CV A13 | 4.9 |
| **75** | 07/2010 | GI | 3.0 y | Stool | *CV A13 | 5.2 |
| **76** | 09/2009 | Healthy | 1.8 y | Stool | CV A17 | 5.5 |
| **77** | 09/2010 | AFI | 1.4 y | Serum | CV A21 | 5.2 |
| **78** | 09/2009 | Healthy | 4.0 y | Stool | PV | 4.9 |
| **79** | 03/2010 | Healthy | 2.7 y | Stool | PV | 4.7 |

CSF = cerebrospinal fluid; CV = coxsackievirus, EV = enterovirus; PV = poliovirus; * = serotype detected in both healthy and sick children; RTI = respiratory tract infection; CNS = central nervous system infection; GI = gastrointestinal infection

*Phylogenetic analysis*

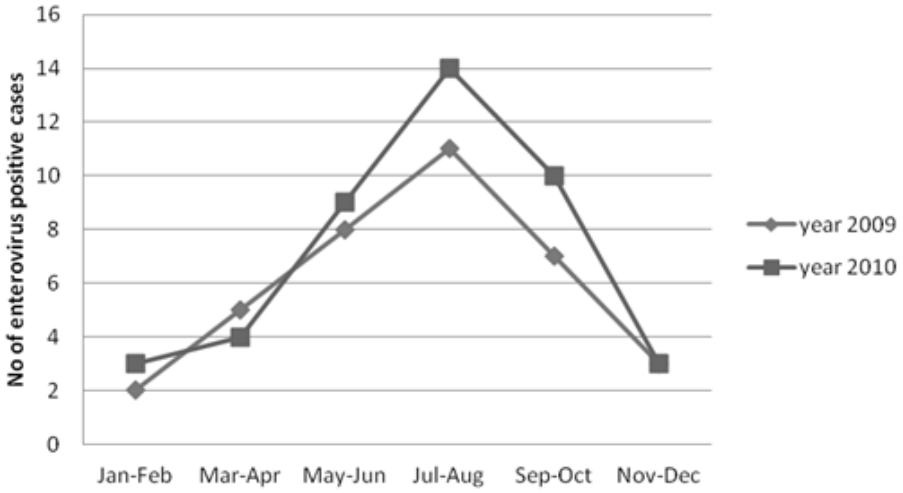
To investigate the genetic relationships between HEV strains from this study and different parts of the world, phylogenetic analysis of partial VP1 region was performed with all corresponding sequences of the respective serotypes in GenBank (dated 10.09.2011). At the nucleotide level, divergence between strains from this study and their reference serotypes ranged from 15% to 25% and within each serotype from 0.5% to 16%. CV B3, CV B5, CV B6, ECV 20 and ECV 29 serotypes were represented by more than one genetic lineage (Figure 2 A-E). The HEV sequences from this study matched most closely with those from America (mainly Georgia isolated during 2002-2005) and European countries in comparison to those from neighbouring Asian countries.

## Discussion

This study describes the prevalence of HEV serotypes circulating in Uttar Pradesh, India, through an unbiased molecular approach. HEV was detected in 24.7% of children, predominantly with high prevalence in the age group of 2 to 3 years (59.49%). This finding is consistent with the observations of Sawyer *et al*. [20] who found that age is significantly associated with clinical manifestations and diseases caused by HEV. A seasonal distribution of enterovirus serotype was found throughout the study with higher detection rates in the rainy (20.5%) and summer (11.4%) seasons than in the winter. The high detection rate of enterovirus during these seasons may be due to the climatic conditions and living standards of the population in this region, which can lead to increased rates of detection [14]. In the current study we identified 32 different enterovirus serotypes, including 15 different enterovirus serotypes observed for the first time in India. The high frequency of HEV serotype detection in this study may be due to high infection pressure in these highly populated areas of India and the adoption of molecular methods specific for the detection of virus in clinical specimens [10].

HEV B was the predominant species(84.8%) detected in both healthy and diseased children. Previous studies have reported that HEV B species are a significant cause of infection in children [21-23]. The high frequency of HEV B species in this region may be responsible for the high rate (62.74%) of clinical manifestations related to the central nervous system (CNS) [12-16]. HEV A species was detected only in symptomatic children (Table), which may be due to the increased virulence of these serotypes in this region [16] and another part of India [24]. Grassly*et al*. [25] reported the silent circulation of poliovirus in healthy children from this region, and during this study we observed a higher frequency of HEV C species detection in healthy children. There are previous reports of recombination between polio virus and HEV C species, which might be responsible for large-scale outbreaks of poliomyelitis [26,27]. Circulation of HEV C species in these highly endemic areas of wild poliomyelitis suggests that enhanced molecular surveillance of enterovirus should be undertaken to prevent any outbreak of poliomyelitis

**Figure 1.** Occurrence of enterovirus cases during 2009 and 2010 in the present study




associated with recombinant vaccine derived poliovirus in the future.

Enterovirus serotypes differ in the pattern of their circulation. At a particular area some serotypes dominate and may evolve into new variants causing an epidemic or outbreak. During this study CV B5 serotype was one of our particular interests because of its predominance in this study, and recently we reported an epidemic of encephalitis associated with CV B5 and ECV 19 from this study area [15]. Previous reports indicate that CV B5 was involved in sporadic cases of neurological diseases with an epidemic pattern of incidence [28-29]. Phylogenetic analysis of CV B5 sequences from this study showed that two genotypes were circulating during this study. The CV B5 strain detected in 2009 comprised one genotype that was clustering with isolates from India associated with encephalitis in this region and from Georgia isolated during 2005 in sewage. The second genotype originated from 2010 strains (Figure 2B) and this finding is comparable with an earlier report of cyclic occurrence of a specific genotype of CV B5 infections in the community [30]. Most of the sequences from this study showed good similarity with American strains (mainly Georgia isolated during 2002-2005) and strains from European countries in comparison with those from neighbouring Asian countries. It might be possible that these serotypes were circulating in different geographical areas [4-5] but may have been missed due to gaps in surveillance from different parts of the world.

## Conclusion

In conclusion, a total of 32 different HEV serotypes were identified including 15 different enterovirus serotypes detected for the first time in India. To our knowledge this is the first report of enterovirus 74 and 80 associated with encephalitis worldwide. Molecular methods of HEV typing and phylogenetic analysis of the partial VP1 gene allow timely detection of the emerging or re-emerging HEV strains and their transmission in different geographical areas.

### Acknowledgements

This study was supported by a grant-in-aid from the Indian Council of Medical Research, Government of India, New Delhi (Ref no: 5/8/7/23/2007-ECD-I).

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**Figure 2A.** Phylogenetic analysis of partial VP1 region of coxsackievirus B3 nucleotide sequences

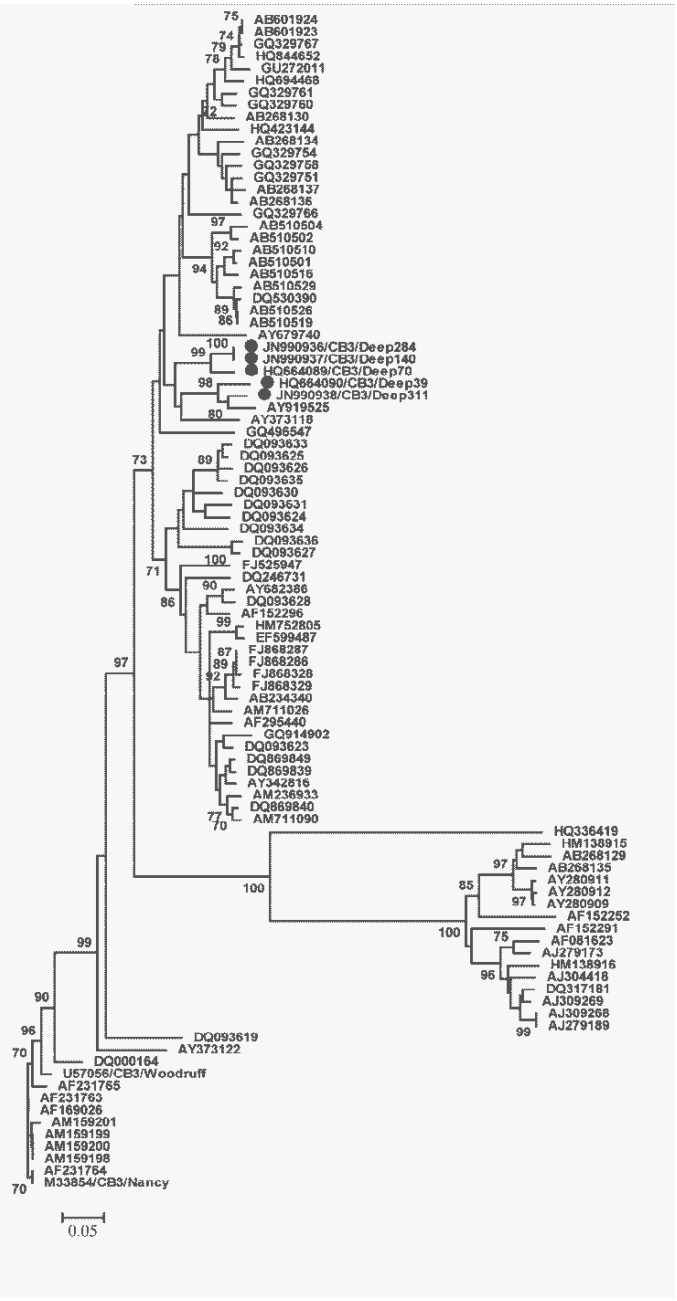
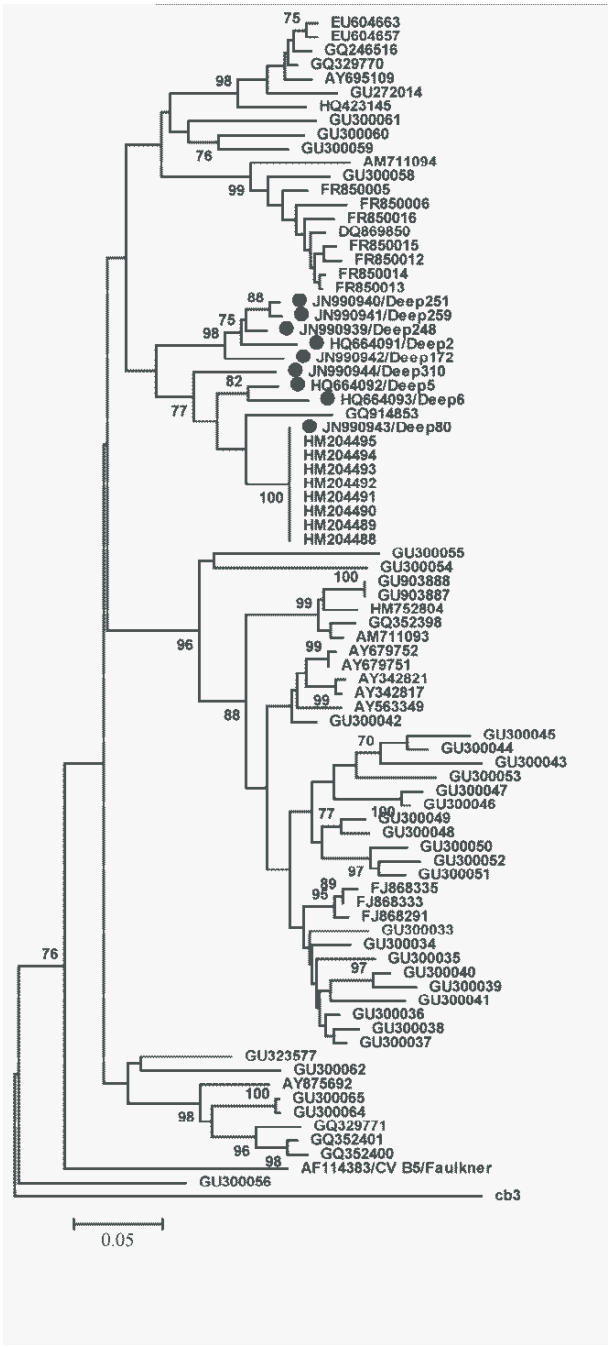



**Figure 2B.** Phylogenetic analysis of partial VP1 region of coxsackievirus B5 nucleotide sequences




Serotypes from this study are designated as a filled circle. Other reference sequences are available from the Genbank database based on the partial VP1 region.Only bootstrap values [70%] are shown. The evolutionary history was inferred using the Neighbor-Joining method by MEGA4 program. Scale bar indicates nucleotide substitutions per site.

Figure 2A and 2B: The tree was constructed by using the neighbour-joining method and Kimura 2-parameter model. Significance of phylogenies was investigated by bootstrap analysis with 1,000 pseudoreplicate datasets. Bootstrap values >70% are indicated on the tree.

**Figure 2C.** Phylogenetic analysis of partial VP1 region of coxsackievirus B6 nucleotide sequences


**Figure 2D.** Phylogenetic analysis of partial VP1 region of echovirus 20 nucleotide sequences

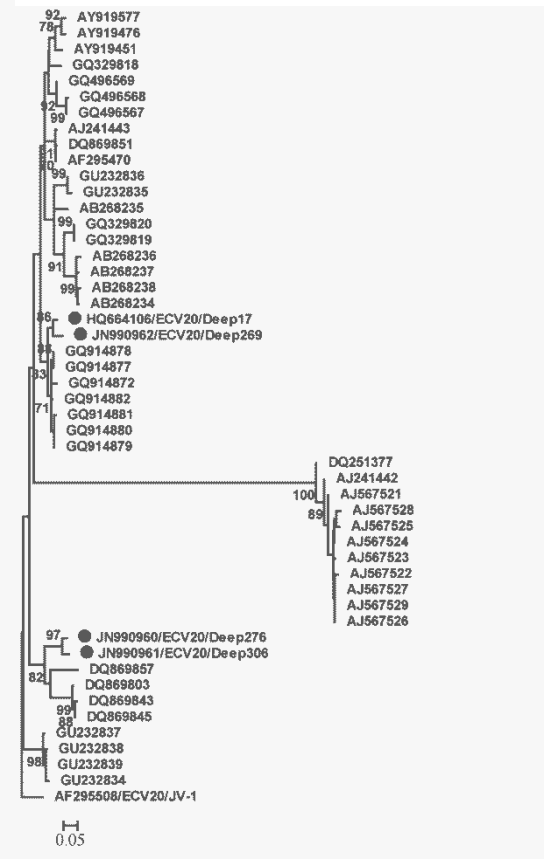
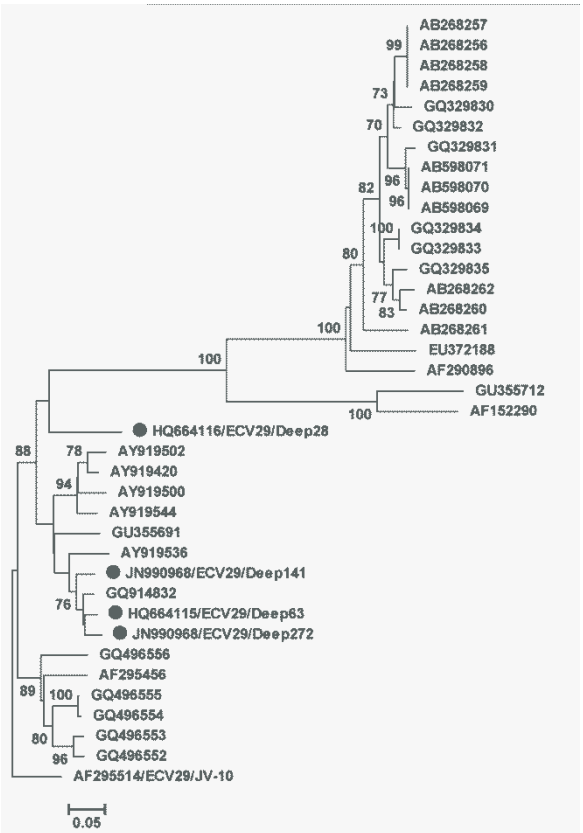



**Figure 2E.** Phylogenetic analysis of partial VP1 region of echovirus 29 nucleotide sequences

Figures 2C, 2D, and 2E: The tree was constructed by using the neighbor-joining method and Kimura 2-parameter model. Significance of phylogenies was investigated by bootstrap analysis with 1,000 pseudoreplicate datasets. Bootstrap values >70% are indicated on the tree.



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**Conflict of interests:** No conflict of interests is declared.