Molecular Characterization of Echovirus 30 Isolated from Environment in North India

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Research

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Abstract

Background

Echovirus 30 (E30) causes acute aseptic meningitis. Enteroviruses (EVs) are responsible for 30,000 to 50,000 hospitalizations for aseptic meningitis per year in the United States. E30 is one of the most frequently isolated EVs, causing extensive outbreaks in temperate climates in several countries.

Methods

E30 used in this study was isolated from environmental specimens. The virus was confirmed by RT-PCR with specific primers (1). Virus stock was prepared by infecting RD cells in 25 cm^2 flasks (Corning Inc. USA). Virus infected and mock infected cells were incubated at 36.5 ºC with 2% MEM. After 48 h of infection, the culture was aliquoted and kept at-80 ºC for further use.

Results

This present work analyzed the E30 genetic diversity in a fragment of 217 nucleotides of the VP1 region, of environmental strains. The environmental samples of echovirus 30 show mutation in the nonfunctional polyprotein protein (Fig. 1). The result suggested that the information may be obtained by analyzing only a part of the VP1 region.

Conclusion

The present study showed that the E30 environmental sample is more divergent to prototype Bastianni strain.

Background

Enteroviruses (EVs) are of public health concern because of the low infectious dose needed to cause disease (2). Enterovirus is nonenveloped, single-stranded, positive-sense viruses belonging to the family Picornaviridae, includes more than 100 serotypes divided among 5 groups (poliovirus, human enterovirus A, human enterovirus B, human enterovirus C, and human enterovirus D) (3, 4, 5) including more than 70 enterovirus serotypes have been identified in humans (6). The genome of EV contains approximately 7,400 nucleotides including a long open reading frame (ORF). It encodes a polyprotein that is cleaved into 4 structural proteins (VP4, VP2, VP3, and VP1) and 7 non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) flanked by a 5'-untranslated region (UTR) and a 3'-UTR (7, 8, 9)

EV may cause various symptoms, varying from asymptomatic infection to fever, hand-foot-and-mouth disease (HFMD) (7), gastroenteritis, myocarditis and aseptic meningitis (10, 11). In the environment, enterovirus can survive under a wide pH range (pH 3 to 10) and for extended periods at low temperature (12).

EVs are responsible for 30,000 to 50,000 hospitalizations for aseptic meningitis per year in the United States. The E30 is one of the most frequently isolated EVs in the United States, comprising 6.8% of all reported EVs isolated from 1970 to 1983 (13) and 9.5% of the EVs isolated from 1993 to 1996 (14). In 1998, E30 accounted for 42% of all the United State.

EV isolations reported to the Centers for Disease Control and Prevention by state and territorial public health laboratories (15). E30 (genus: Enterovirus; family, Picornaviridae) is one of the most frequently isolated EVs, causing extensive outbreaks of E30 in temperate climates in several countries (15, 16). Additionally, this serotype is one of several enteroviruses associated with sporadic cases of the aseptic meningitis (17). In contrast to the highly conserved 5’ UTR, the open reading frame encoding capsid protein VP1 is more variable and confers distinct antigenic properties of the virus. Thus, this VP1-encoding region of the genome is considered most suitable for sequence analysis, and to determine the Enterovirus genotype and genetic variation (18). In this study, we tried to analyze the E30 (an environmental strain) by RT-PCR and sequencing of the VP1 gene allowed us to understand its genetic diversity.

Materials And Methods

Sewage sample collection

Sewage sample (approx one liter) was collected and stored in sterile glass bottle from the collection site by directly dipping into the sewage effluent. The outer surface of the bottle was rinsed with 2% sodium hypo chloride solution, placed in a cool box, and transported to the laboratory. The bottle was frozen at − 20° c, until the process of virus isolation. This procedure is already reported in our previously published article (11).

Sewage sample processing

Sewage sample was concentrated by two-phase concentration method described previously (11). In brief, the pH of the sample was adjusted to 7.2 and the sample was centrifuged at 5000 g for 30 min at 4 °C to pellet the solids. 500 ml of clarified sewage was mixed with defined amounts of two polymers, dextran and 15% polyethylene glycol (15% PEG6000). The homogenous mixture obtained by vigorous shaking is left to stand overnight at 4° C in a separating funnel. This allows the polymers to separate in two distinct layers (phases) in the funnel. Enterovirus accumulates in the smaller bottom layer and/or at the boundary between the layers (interphase). The bottom layer and interphase were collected drop-wise. The pellet from the initial centrifugation was suspended in this concentration, and treated with chloroform, then centrifuged at 5000g for 30 min at 4°C and assayed for the presence of virus. The concentrated virus suspension was stored at − 20°C until used for virus isolation (19). This method was published in a study which compared three different methods, including direct isolation, centrifugation and two phase separation, and results of their study suggest that the two phase separation method is the best for maximum virus yield (20).

Inoculation of sewage samples

The extracted concentrate was inoculated on fresh monolayer cultures of L20B and RD cells in 50 ml (25 cm2) flasks. The cultured flasks were incubated at 37 °C and examined at every 24 h for cytopathic effect (CPE). Samples which showed CPE were frozen and thawed two times, then re-passaged on new cells. Samples were observed for CPE up to 7 days before being considered negative. Samples showed CPE were stored for confirmation through serotyping.

Cell culture

Human rhabdomyosarcoma (RD) and L20B (transgenic) cells were obtained from the Center for Disease Control and Prevention, Atlanta, GA, USA. Minimum essential media (MEM) of Earle's salt solution and fetal bovine serum (FBS) were purchased from (Sigma Aldrich, USA). All cell culture media contained HEPES buffer, L-glutamine, sodium bicarbonate penicillin, streptomycin, amphotericin B from GIBCO, USA. Cell cultures were grown at 37 °C in incubators with supply of 5% CO2. Cell cultures for virus isolation were grown in 25cm2 plastic flasks (Costar, Corning, N.Y.) with 10% FBS (MEM) and maintained at 2% FBS (MEM) containing 7 ml MEM, and tissue culture tube with 1.5 ml (2% FBS).

Virus culture and Serotyping

E30 used in this study was isolated from environmental specimens. Virus stock was prepared by infecting RD cells in 25 cm^2 flasks (Corning Inc. USA). Virus infected and mock infected cells were incubated at 36.5 ºC with 2% MEM. After 48 h of infection, the culture was aliquoted and kept at-80 ºC for further use. The virus was serotyped according to World Health Organization's protocol (21) and confirmed by RT-PCR with specific primers (1).

RT-PCR

Isolates grown in the RD cells were freeze-thawed three times and centrifuged at 12000 rpm for 10 minutes. The 5µl isolate supernatant was diluted with 10µl of distilled water. Primer E-1 and E-2 were used 50 pmol concentration as described previously (21, 1), the buffer containing 100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl (pH 8.3), 1 µL of diluted samples were added to the appropriate tube in the Bio-safety cabinet. The tubes were incubated at 95 ºC in the thermocycler for 5 min. and immediately chilled on ice followed by addition of 5 µl enzyme buffer containing 0.7 µl of 1M DTT (dithiothreitol), 6.9 µL of 40 U $µl^{-1}$ Protector RNase inhibitor, 4.5 µl of 20 U $µl^{-1}$ of the Avian Myeloblastosis Virus (AMV) Transcriptase, 13.7 µl of 5 U $µl^{-1}$ Taq polymerase and 1.0 µl of 10 mM dNTPs. PCR tubes were placed in the thermocycler in the RT reaction at 42 ºC for 20 min. and for inactivation at 95 ºC for 3 min. For PCR amplification, 30 cycles of denaturation at 95 ºC for 45 sec, annealing at 55 ºC for 45 sec, and extension at 70 ºC for 45 sec, followed by cooling at 4 ºC has been used.

RT-PCR product purification

DNA was eluted from gel and has been purified by the QIAGEN gel extraction columns (QIAGEN, Chatsworth, CA, USA). PCR products were examined and randomly five E30 isolates selected for sequencing.

Sequencing reaction

Sequencing reaction mix was contained, 4 µl of big dye terminator ready reaction mix, 2 µl of each primer (10 pmol λ^{-1}), 3 µl Milli-Q water and 1µl of template (100 ng μl^{-1}). PCR conditions followed (25 cycles) initial denaturation at 96 °C for 1min, denaturation at 96° C for 10 sec., hybridization at 50 °C for 5 sec. and elongation at 60 °C for 4 min. The ABI 3130 genetic analyzer and chemistry big dye terminator version 3.1 cycle sequencing kit were used for sequencing. Polymer and capillary array used POP_7 polymer 50 cm capillary array and BDTv3-KB-Denovo_v 5.2 protocol were examined, whereas data were analyzed by software Seq Scape_v 5.2 and reaction were examined in the Applied biosystem micro Amp optical 96-well reaction plate.

Construction of phylogenetic trees

Nucleotide BLASTn analysis (http://www.ncbi.nlm.nih.gov/BLAST) was used to identify related genes of the viruses and construct the phylogenetic tree. The reference sequences were obtained from the GenBank. The ClustalX version 2.0.12 (22) was used to perform multiple nucleotide alignment and tree was constructed (Njplot Version 2.3) (23), the neighbor-joining method according to the distances between all pairs of sequences in a multiple alignment. The confidence of sequence clustering was evaluated by bootstrapping (1000 replicates) (24, 11) in fig.1.

Results

There were 109 sewage samples were collected for this study. 44 out of 109 were detected positive for enterovirus in sewage samples by cell culture and followed by serotyping and RT- PCR. The existence of echovirus was observed throughout the year however the peak season for the maximum detection of other EV was from July to September in each year.

Sewage isolates analysis

Human echoviruses- 47.72% (25), coxsackieviruses B- 11.4% (26), PV- 15.9% (12) and Untypeable- 25.0% (19) were found out of 44 EV positive isolates. During the month of July to September NPEV and UT were predominant, which accounted for 83.3 and 16.6% respectively, Untyped samples were analyzed by RT-PCR using pan-EV primers (CDC, Atlanta, GA) and E30 followed by sequencing. NPEV serotypes showed 95 to 100% homology with other enteroviruses (Fig. 1).

Isolation and typing

The one isolate was isolated from RD cells. The one strain was confirmed to be E30 by the amplification of the partial VP1 sequences. The sequence was analysed using an online enterovirus genotyping tool.

This was isolated from environmental sample.

VP1 sequence analysis

This present work analyzed the E30 genetic diversity in a fragment of 217 nucleotides of the VP1 region, of environmental strains. The environmental samples of echovirus 30 show mutation in the nonfunctional polyprotein protein (Fig. 1). The result suggested that the information may be obtained by analyzing only a part of the VP1 region. The partial VP1 sequence of the one strain had the highest similarity with other E30 strains in the GenBank database and had a percentage reaching 98%. The isolated strain was identified as E30 using an EV serological and molecular typing criteria.

Nucleotide sequence accession number

The accession number of the partial VP1 nucleotide sequence of the E30 strain identified in this study is GQ353352.

Discussion

Molecular mechanisms of picornavirus variation and evolution result from point mutations and genomic rearrangement, in particular recombination. Although most attention has been directed towards mutation, recent studies have indicated that recombination might have an important role in enteroviruses. The genetic recombination was involved in a time-correlated manner in their emergence and that drift occurred in all lineages, quasi exclusively by synonymous nucleotide substitutions, indicating strong constraints against amino acid changes in both structural and non-structural genes (27). This pattern of evolution is clearly different from that of other enteroviruses. A single lineage at a time appears to be circulating worldwide. This behavior may be related to the epidemic activity of the E30.

Partial sequencing in the VP1 gene was previously proved to be suitable for serotype identification of EVs (28, 29, 30, 15, 31), a step which precedes phylogenetic analysis (Fig. 1). This molecular approach for serotyping was proposed to be used in routine diagnosis instead of the laborious and time-consuming seroneutralization assays. For phylogenetic analysis study the VP1 region for several reasons. It is one of these regions of the VP1 that proved to discriminate well between EV serotypes, the use of the same PCR product and sequence data for both serotype identification and phylogenetic analysis, being of great interest on the practical level (28). The 3’end of the VP1 has been extensively used in the molecular epidemiology of poliovirus (32) and echovirus 30 (33, 34) and proved to be appropriate to discriminate EV isolates in Geno-groups, genotypes and lineages. Many authors have previously used this part of the VP1 for molecular studies of E30; the number of sequences available in the international database, matching the 217 nucleotides considered herein, is higher in comparison to the other parts of the VP1. E30 is among the most commonly isolated EVs in the world. The E30 may cause a full range of EV diseases; some being of particular importance, like severe diseases in neonates and aseptic meningitis with epidemic potential. However, studies on the molecular epidemiology of this infection have been limited to few countries in the world. The present work showed that the genetic characteristics of E30 circulating

strains and the dynamics of genetic evolution may differ from one country to another according to their geographical location and endemicity levels. It also contributes to a better understanding of the epidemiology of the E30.

Conclusion

It could be concluded that the E30 environmental sample is more divergent to prototype Bastianni strain, and the phylogenetic analysis of E30 strain revealed an evolutionary change. This study is also helpful to those who are struggling to eradicate Polio virus in term of better understanding of its presence in community.

Abbreviations

AMV: Avian Myeloblastosis Virus

CPE: Cytopathic effect

DTT: Dithiothreitol

E30: Echovirus 30

EVs: Enteroviruses

FBS: Fetel bovine serum

HFMD: Hand-foot-and-mouth disease

MEM: Minimum essential media

ORF: Open Reading Frame

PEG: Polyethylene glycol

RD: Rhabdomyosarcoma

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data available in this article is supporting the results reported in a published article can be found online (Pubmed, Medline or Google search).

Competing interests

The authors declare that there is no competing interest regarding the publication of this article.

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Authors' contributions

Both authors contributed equally to this research work and writing of the manuscript.

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References

1. Kilpatrick, D.R., Quay, J., Pallansch, M.A., Oberste, M.S. Type-specific detection of echovirus 30 isolates using degenerate reverse transcriptase PCR primers. J Clin Microbiol 2001; 39 (4), 1299-1302. doi:1128/JCM.39.4.1299-1302.2001.
2. Haas CN, Rose JB, Gerba C, Regli S. Risk assessment of virus in drinking water. Risk Anal. 1993; 13(5):545-52. doi: 10.1111/j.1539-6924.1993.tb00013.x.
3. Pallansch MA, Roos RP. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Knipe DM, Howley PM, eds. Fields Virology. 4th ed. Philadelphia: Lippincott Williams and Wilkins: 2001; 723-75.
4. Rishi K. Singh, Sarika Tiwari, Vijaya L. Nag, Raj K. Singh, Tapan N. Dhole. Evolution In Partial Sequences Of Serological Variants Affects The 5’ Utr Of Echovirus 9 And 11. International Journal of Pharmacology & Toxicology2012;/ 2(2), 2012, 83-94. Corpus ID: 86072342
5. Gupta A, Robert W. Tolan, Asymptomatic (Subclinical) Meningitis in One of Premature Triplets with Simultaneous Enteroviral Meningitis: A Case Report. AJP Rep. Nov 2012; 2(1): 15–18. doi: 1055/s-0031-1296029.

6. Ying-Han C, Wen Li D, Marne Cet al. Phosphatidylserine Vesicles Enable Efficient En Bloc Transmission of Enteroviruses. Cell. 2015 Feb 12;160(4):619-630. doi: 10.1016/j.cell.2015.01.032.
7. Hongbo Liu, Jie Zhang, Yilin Zhao, Haihao Zhang, Keqin Lin, Hao Sun, Xiaoqin Huang, Zhaoqing Yang & Shaohui Ma. Molecular characterization of echovirus 12 strains isolated from healthy children in China. SCIentIfIC ReportS 2018;11716, doi:10.1038/s41598-018-30250-x
8. Song, Y. et al. Phylogenetic Characterizations of Highly Mutated EV-B106 Recombinants Showing Extensive Genetic Exchanges with Other EV-B in Xinjiang, China. Sci Rep-Uk. 7, 43080, 2017; https://doi.org/10.1038/srep43080.
9. Nasri, D. et al. Basic Rationale, Current Methods and Future Directions for Molecular Typing of Human Enterovirus. Expert Rev Mol Diagn. 7, 2007; 419–434, https://doi.org/10.1586/14737159.7.4.419.
10. Melnick JL. New picornavirus vaccines for hepatitis A, and lessons from the control of poliomyelitis by the prototype picornavirus vaccines. Prog Med Virol, 1990; 37:47- 55.
11. Sarika Tiwari and Tapan N. Dhole. Assessment of enteroviruses from sewage water and clinical samples during eradication phase of polio in North India. Virology Journal 2018; 15:157. org/10.1186/s12985-018-1075-7.
12. Kocwa-Haluch R. Water borne enteroviruses as a hazard for human health. Pol J Environ Stud. 2001; 10:485–7.
13. Strikas, R.A., Anderson, L.J., Parker, R.A. Temporal and geographic patterns of isolates of nonpolio enterovirus in the United States, 1970-1983. J Infect Dis 153, 346-51. doi: 10.1093/infdis/153.2.346.
14. CDC and Prevention. 1997. Nonpolio enterovirus surveillance—United States 1993–1996., Morbid. Mortal. Wkly. Rep.1997; 46(32); 748-750.
15. Oberste, M.S., Maher, K., Kennett, M.L., Campbell, J.J., Carpenter, M.S., Schnurr, D., Pallansch, M.A. Molecular epidemiology and genetic diversity of echovirus type 30 (E30): genotypes correlate with temporal dynamics of E30 isolation. J Clin Microbiol 1999a; 37, 3928-33. PMID: 10565909.
16. Trallero, G., Casas, I., Tenorio, A., Echevarria, J.E., Castellanos, A., Lozano, A., Brena, P.P. Enteroviruses in Spain: virological and epidemiological studies over 10 years (1988-97). Epidemiol Infect 2000; 124, 497-506. doi: 1017/s0950268899003726.
17. Dagan, R., Jenista, J.A., Menegus, M.A. Association of clinical presentation, laboratory findings, and virus serotypes with the presence of meningitis in hospitalized infants with enterovirus infection. J Pediatr 1988; 113, 975-8.
18. Wang, J.R., Tsai, H.P., Huang, S.W., Kuo, P.H., Kiang, D., Liu, C.C. Laboratory diagnosis and genetic analysis of an echovirus 30-associated outbreak of aseptic meningitis in Taiwan in 2001. J Clin Microbiol. 2002; 40, 4439-44. doi: 1128/jcm.40.12.4439-4444.2002.
19. World Health Organization. Expanded Programme on Immunization. Guidelines for environmental surveillance of poliovirus circulation.WHO/V&B/03.03 Geneva, Switzerland. 2003; also available at apps.who.int/iris/bitstream/10665/67854/1/WHO_V-B_03.03_eng.pdf

20. Kargar M, Sadeghipour S, Nategh R. Environmental surveillance of Non-Polio Enteroviruses in Iran.Virol J. 2009; 6:149. org/10.1186/1743-422X-6-149.
21. Tiwari S, Singh RK, Bharti S, Roy R, Singh RK, Dhole TN. An in vitro study- Indian strain of Japanese encephalitis virus infection in porcine stable kidney cell using 1H NMR spectroscopy. International Journal of Experimental Pharmacology. 2012; 2(2):50–8. e-ISSN 2248 - 9169
22. Thompson, J.D., Higgins, D.G., Gibson, T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994; 22, 4673-80. doi: 10.1093/nar/22.22.4673.
23. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evolution. 1987; 4:406–25. doi: 10.1093/oxfordjournals.molbev.a040454.
24. Felsenstein, J. 1993. PHYLIP: Phylogeny inference package, version 3.5 c. Distributed by Department of Genetics, University of Washington. Seattle, USA.
25. Gregory JB, Litaker RW, Noble RT. Rapid one-step quantitative reverse transcriptase PCR assays with competitive internal positive control for detection of enteroviruses in environmental samples. Appl Environ Microbiol. 2006; 72(6):3960–7. doi: 1128/AEM.02291-05.
26. Melnick JL. New picornavirus vaccines for hepatitis a, and lessons from the control of poliomyelitis by the prototype picornavirus vaccines. Prog Med Virol. 1990; 37:47–55.
27. Mirand, A., Henquell, C., Archimbaud, C., Peigue-Lafeuille, H., Bailly, J.L. Emergence of recent echovirus 30 lineages is marked by serial genetic recombination events. J Gen Virol 2007; 88, 166-76. org/10.1099/vir.0.82146-0
28. Caro, V., Guillot, S., Delpeyroux, F., Crainic, R. Molecular strategy for 'serotyping' of human enteroviruses. J Gen Virol. 2001; 82, 79-91. doi: 10.1099/0022-1317-82-1-79.
29. Norder, H., Bjerregaard, L., Magnius, L.O. Homotypic echoviruses share aminoterminal VP1 sequence homology applicable for typing. J Med Virol. 2001; 63, 35-44. doi: 1002/1096-9071(200101)63:1<35::aid-jmv1005>3.0.co;2-q
30. Oberste, M.S., Maher, K., Flemister, M.R., Marchetti, G., Kilpatrick, D.R., Pallansch, M.A. Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. J Clin Microbiol 2000; 38, 1170-4. doi: 1128/JCM.38.3.1170-1174.2000.
31. Oberste, M.S., Maher, K., Kilpatrick, D.R., Flemister, M.R., Brown, B.A., Pallansch, M.A. Typing of human enteroviruses by partial sequencing of VP1. J Clin Microbiol 1999b; 37, 1288-93. doi: 1128/JCM.37.5.1288-1293.1999.
32. Kew, O., Mulders MN, Lipskaya GY, da Silva EE, Pallansch MA. Molecular epidemiology of polioviruses. Semin Virol. 1995; 6, 401-414. org/10.1016/S1044-5773(05)80017-4.
33. Kunkel, U., Schreier, E. Genetic variability within the VP1 coding region of echovirus type 30 isolates. Arch Virol 2000; 145, 1455-64. doi: 1007/s007050070102
34. Savolainen, C., Hovi, T., Mulders, M.N. Molecular epidemiology of echovirus 30 in Europe: succession of dominant sublineages within a single major genotype. Arch Virol 2001; 146, 521-37. doi: 10.1007/s007050170160.

Figures

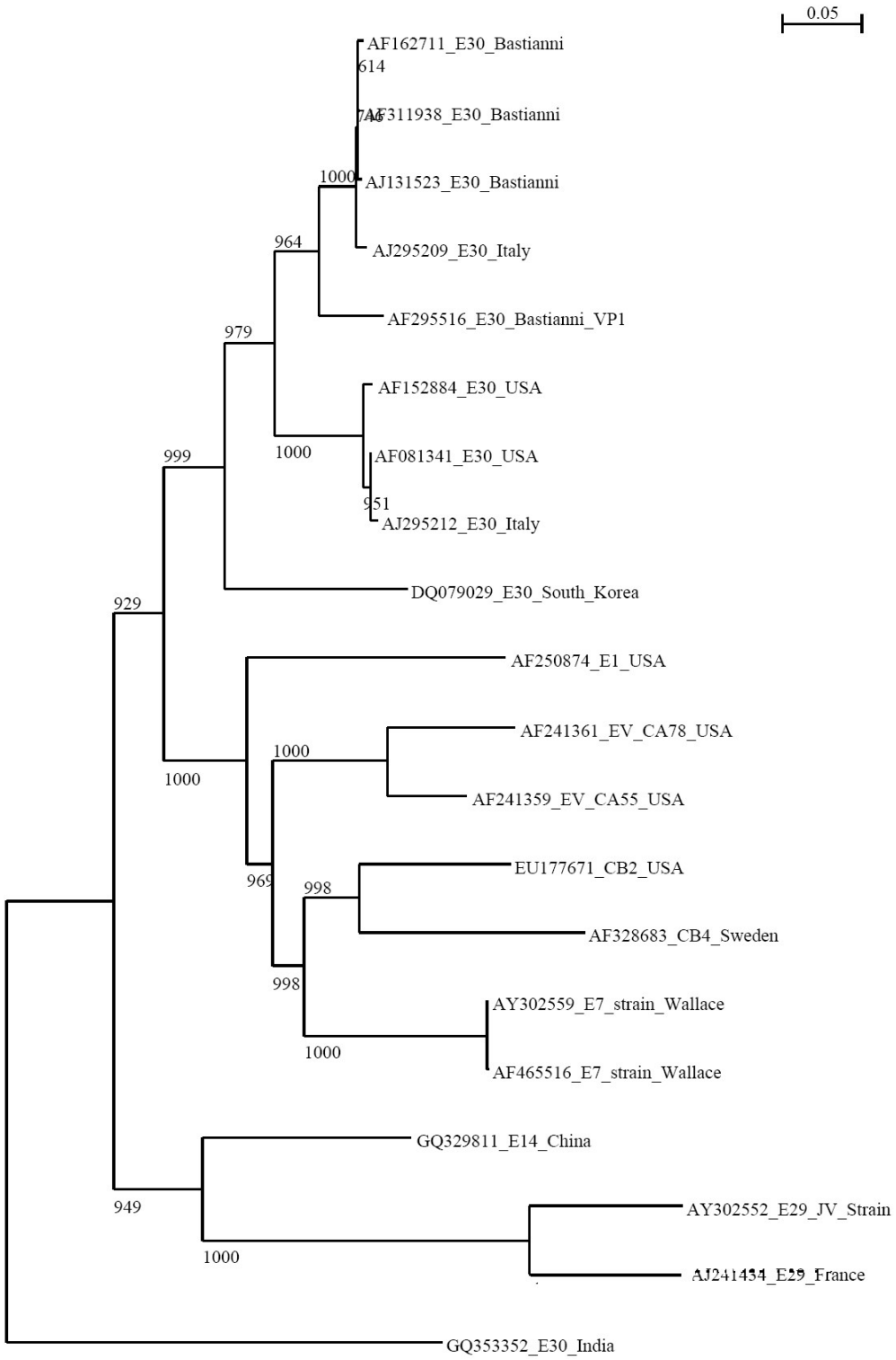

Figure 1

Phylogenetic tree based on DNA distance inferred from Indian E30 isolate sequence bank accession no. GQ353352 (All HEV isolates sequences available in NCBI sequence database).