

Enterovirus 71 isolated from a case of acute flaccid paralysis in India represents a new genotype

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Enterovirus 71 is the causative agent of a variety of diseases ranging from the mild hand-foot-and-mouth-disease to severe neurological complications like meningitis, encephalitis and polio-like paralysis. In recent years, large-scale outbreaks with many fatal cases due to enterovirus 71 infections have occurred in Asia-Pacific region. Hand-foot-and-mouth-disease or neurological disease manifestations of enterovirus 71 etiology have not been recognized in India. Enterovirus 71 was isolated in our laboratory from one case of acute flaccid paralysis (Guillain-Barré syndrome) from the state of Haryana reported through the acute flaccid paralysis surveillance system that was established in India since 1997. No additional enterovirus 71 isolates were found among non-polio enteroviruses isolated from 101 other cases of acute flaccid paralysis having residual weakness 60 days post-onset from the same geographical region. Nucleotide sequence analysis of the 5' non-translated region and capsid proteins VP1 and VP4 show that the Indian isolate is genetically distinct from enterovirus 71 strains isolated during the recent outbreaks in the Asia-Pacific region. Emergence of enterovirus 71 in India needs to be closely monitored.

ENTEROVIRUS 71, a member of genus Enterovirus of virus family Picornaviridae causes a variety of neurological diseases including aseptic meningitis, encephalitis and poliomyelitis-like paralysis^{1,2}. Enterovirus 71 is also one of the few enterovirus serotypes often associated with hand-foot-and-mouth-disease, a mild childhood exanthema³. Since its discovery, sporadic cases and outbreaks of infection of enterovirus 71 have been reported from many parts of the world⁴⁻¹². Four large outbreaks of enterovirus 71 infection with severe neurological complications and fatal cases have occurred, viz. Bulgaria, Hungary, Malaysia and Taiwan. In Bulgaria, the disease presented clinically and pathologically all known forms of poliomyelitis resulting in 44 fatal cases. In Sarawak at least 29 children suffering from hand-foot-and-mouth-disease caused by enterovirus 71 died of rapidly progressive cardiopulmonary failure^{13,14}. 405 cases of severe neurological disease and 78 fatal cases of brainstem encephalitis and neurogenic pulmonary oedema were reported during the hand-foot-and-mouth-disease outbreak

in Taiwan in 1998. Enterovirus 71 infections continued to be reported from the Asia Pacific region in 2000–2001.

Enterovirus 71 is genetically most closely related to coxsackievirus A16, the other common cause of hand-foot-and-mouth-disease. Phylogenetic analysis of complete VP1 sequences of enterovirus 71 isolates has led to identification of three genotypes¹⁵. Genotype A is represented by only one isolate (BrCr-CA-70). Two genetic lineages within B and C genotypes (B1, B2 and C1, C2) showed worldwide distribution. Enterovirus 71 strains isolated during recent outbreaks in Asia Pacific region belonged to new sub-lineages within B and C genotypes¹⁶. Comparison of complete genomic sequences of EV71 with poliovirus sequences failed to detect any correlate for the neurovirulence of the virus¹⁷.

Epidemiology of enterovirus 71 has remained unknown in India. Outbreaks of hand-foot-and-mouth-disease have not been reported from India. Cases of encephalitis, aseptic meningitis or polio-like paralysis of enterovirus 71 etiology have not been recognized so far. Since 1997, surveillance of acute flaccid paralysis was established in India to support polio eradication program¹⁸. More than 5000 cases of acute flaccid paralysis occur in India annually. A network consisting of WHO-accredited laboratories performs virological studies to detect infection by enteroviruses¹⁹. Cases of acute flaccid paralysis due to wild poliovirus infection are of primary importance to the polio eradication program. Non-polio enteroviruses isolated from the cases are normally not studied further.

With the objective to determine non-polio enterovirus types of epidemiological significance, a study was undertaken to identify non-polio enteroviruses isolated from cases of acute flaccid paralysis having residual weakness beyond 60 days of onset of paralysis. We detected enterovirus 71 from one case of Guillain-Barré syndrome. Considering the importance of enterovirus 71 in public health, in this communication we present details of the case and molecular characterization of the virus isolate.

A 5-year-old boy from Faridabad district, Haryana was admitted to the Safdarjung Hospital, New Delhi on 5 July 2001 due to onset of acute flaccid paralysis (date of onset 2 July 2001). The patient presented with ascending symmetrical quadriplegia that reached maximum deficit in 2 days. There was no fever at onset of paralysis as well as no sensory loss. Cerebrospinal fluid (CSF) analysis revealed a mild albumino-cytological dis-association. Nerve conduction velocity test done on 7 July 2001 showed motor neuropathy involving the right side only. At the time of follow-up examination carried out 60 days after onset of paralysis, hypotonia and areflexia persisted and power of all muscle groups was grade II to grade III with no asymmetrical wasting. A second follow-up examination conducted 11 months after onset showed residual weakness in distal muscles consistent with Guillain-Barré syndrome.

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Suspensions of stool specimens (6th and 13th day post-paralysis) were inoculated in RD (human rhabdomyosarcoma) cell line for virus isolation²⁰. Both the specimens (R13222 and R13223) produced cytopathic effect in RD cell cultures. The virus isolates were identified as Enterovirus 71 by neutralization test using rabbit anti-Enterovirus 71 (BrCr) antiserum prepared earlier in the authors' laboratory.

VP1 nucleotide sequences have been successfully used for serotype identification of enterovirus²¹. Primer pair (292/222) that amplifies a 358 bp fragment (nucleotides 2612 to 2969, relative to Poliovirus type 1, Mahoney) of VP1 of enteroviruses was used to amplify RNA extracted from the isolate R13223 in a reverse transcription-polymerase chain reaction as described by Oberste *et al.*²¹. The amplified segment was sequenced using BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Bio-systems, Foster City, CA, US) and the protocol supplied by the manufacturer on an automated DNA sequencer Model 310 (Applied Bio-systems). Both the forward and reverse strands of the amplified cDNA were sequenced. The partial VP1 sequence of R13223 was subjected to BLAST analysis to search for closely similar sequences in the database. BLAST analysis returned 'Enterovirus 71' as the closest match confirming results of serological typing of R13223.

Nucleotide sequencing of 5' non-translated region (5'NTR), VP1 and VP4 regions of R13223 was carried out for defining genetic relationship with enterovirus 71 isolates from other countries. Primers used for specific amplification of overlapping segments of enterovirus 71 RNA were as per Brown *et al.*¹⁵, viz., primer pair 340/170 (5'NTR nt 27 to 533), 171/286 (5'NTR-VP2 nt 470 to 1274), 159/162 (VP3-VP1, nt 2385 to 2869), 161/NP1A (VP1 2766 to 3355). The primers used were obtained from M. A. Pallansch, CDC, Atlanta, Georgia, US. RT-PCR and sequencing was carried out as described in the previous section. Nucleotide sequences of the Indian isolate were submitted to GenBank under accession nos. AY179600 to AY179602. Previously published enterovirus 71 sequences used in comparison of the isolate (R13223) were obtained from GenBank database.

VP1 gene of enterovirus 71 comprises 891 nucleotides. VP1 gene sequence (nucleotide nos. 2442 to 3282, 841 nucleotides) of the isolate R13223 was compared with sequences of 30 isolates from different parts of the world. These isolates represent currently known genotypes and the different lineages of enterovirus 71 including those associated with recent outbreaks in Malaysia, Taiwan and Western Australia. Phylogenetic analysis of VP1 sequences is presented in Figure 1. The neighbour-joining tree was constructed using maximum likelihood distances calculated by the program *Tree Puzzle* and support for internal branches of the tree topology was obtained from 1000 puzzling steps²². Our analysis has identified the enterovirus 71 genotypes A, B and C as well as the newer line-

ages represented by isolates from Malaysia, Singapore, Australia and Taiwan within the genotypes B and C. The genetic distance of strain R13223 from each of the genotypes A, B and C exceeds 20% within the VP1 gene. Amino acid sequence, of VP1 protein was deduced from the nucleotide sequence. Though R13223 has a substantially different nucleotide sequence, no unique amino acid substitutions were found. R13223 does not contain the alanine to valine substitution (Ala → Val) at position 170 detected in enterovirus 71 isolates associated with neurological disease in Western Australia¹⁶.

5'NTR of enterovirus 71 comprises 743 nucleotides. Phylogenetic analysis of 5'NTR nucleotide sequence of R13223 (nucleotide nos.130 to 735, 606 nucleotides) also shows that the Indian isolate belongs to a completely different lineage of enterovirus 71 (Figure 2). Similar conclusion was drawn from sequence analysis of VP4 gene (data not shown).

Genetic analysis of capsid protein genes (VP1 and VP4) and the 5'NTR of the isolate (R13223) reveal that the Indian strain of enterovirus 71 represents a lineage completely different from those detected among recent isolates from Malaysia, Singapore, Western Australia and Taiwan as well as the older isolates from European countries and the US. Thus the Indian isolate has no genetic relationship with enterovirus 71 involved in the outbreaks in the Asia-Pacific countries. Enterovirus isolates showing more than 15% sequence diversity in VP1 gene constitute separate genotypic groups²³. The Indian isolate, therefore, represents a new genotype of enterovirus 71. We propose 'genotype D' designation to the Indian isolate of enterovirus 71.

Since 1997, an efficient surveillance system to detect cases of acute flaccid paralysis has been established under the polio eradication programme¹⁸. With good-quality AFP surveillance system established in the country, there is no reason to believe that outbreak(s) of CNS disease would go unnoticed.

The enterovirus 71 isolate reported in this communication was initially reported as a non-polio enterovirus and the case was classified as 'AFP due to non-polio causes', final clinical diagnosis being Guillain-Barré syndrome. The non-polio enterovirus isolate was identified as enterovirus 71 when typing of isolates from cases of acute flaccid paralysis with residual weakness (60 days post paralysis) was undertaken. We studied non-polio enteroviruses isolated from 101 additional cases of acute flaccid paralysis having residual weakness beyond 60 days of onset of paralysis reported during 2000 to June 2002 from the same geographic area. Enterovirus 71 was not detected among these non-polio enteroviruses.

As a general property of most enteroviruses, a severe disease appears to be a rare consequence of a relatively common infection. However, several large epidemics of EV71 infection have occurred in Asia-Pacific region since 1997. Hundreds of cases of HFMD were reported

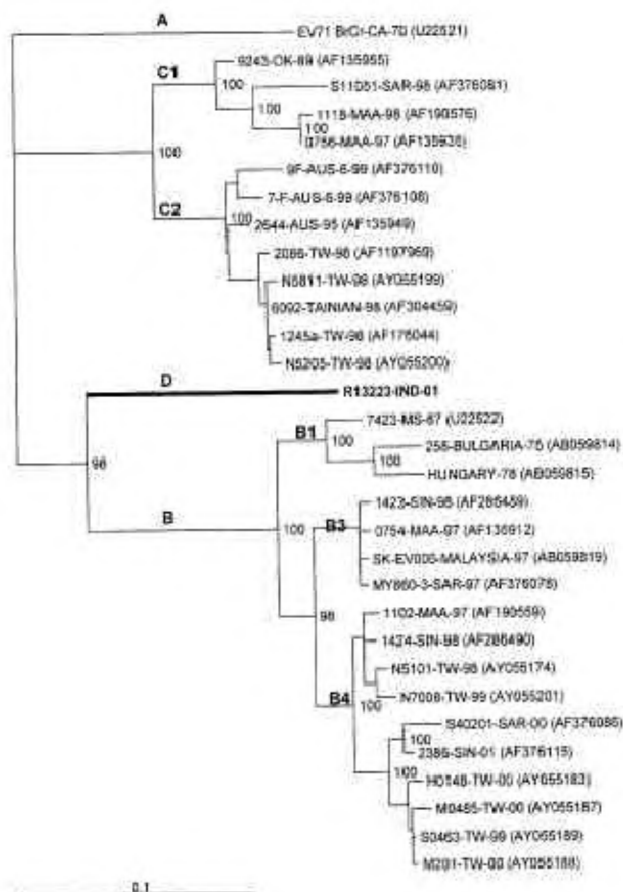


Figure 1. Phylogenetic tree showing genetic relationship of enterovirus 71 isolate R13223-IND-01 and strains representing currently known genotypes based on comparison of VP1 nucleotide sequences (nt 2442 to 3282). The neighbour-joining tree was constructed using *Tree Puzzle*. Support for internal branches of the tree topology shown as percentages at the nodes were obtained from 1000 puzzling steps. Strain identification and GenBank accession numbers denote sequences obtained from GenBank. Letters written on the branches indicate genotype and lineage designations. The scale indicates relative phylogenetic distance.

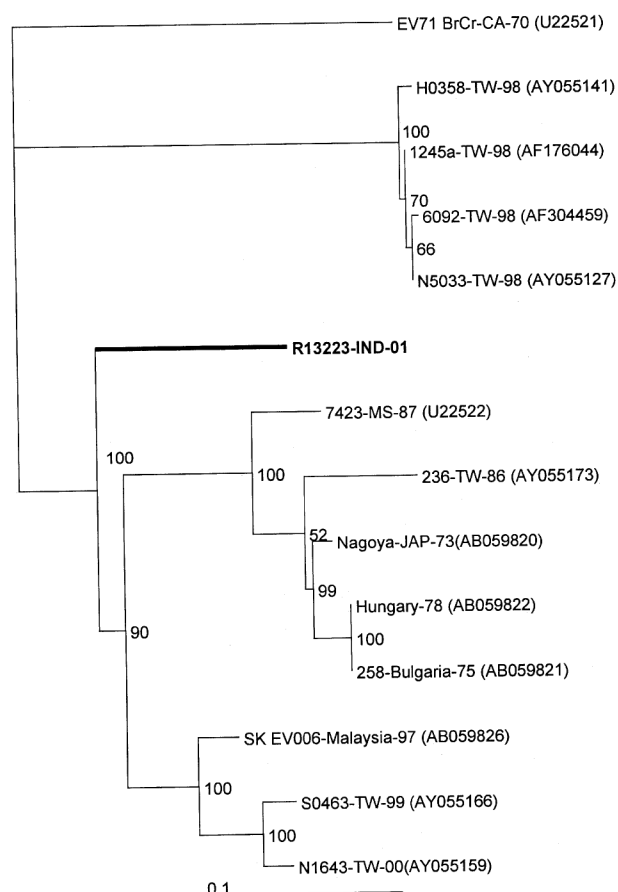


Figure 2. Phylogenetic tree showing genetic relationship of enterovirus 71 isolate R13223-IND-01 and strains representing currently known genotypes based on comparison of 5' non-translated region nucleotide sequences (nt 130 to 606). Strain identification and GenBank accession numbers denote sequences obtained from GenBank. Details of tree reconstruction are as per Figure 1.

with neurological complications arising in a small proportion of cases^{4,11,24}. Clusters of deaths of patients have occurred during the epidemics of hand-foot-and-mouth-disease in Sarawak, Malaysia and Taiwan. No correlation between severity of disease and genetic lineage of enterovirus 71 has been detected within the VP1 region of the genome¹⁵. The reasons this common pathogen caused large outbreaks in Sarawak, Malaysia and Taiwan with many fatalities and severely handicapped survivors remain unknown. There have been no outbreaks of hand-foot-and-mouth-disease in India and in our study only one case of enterovirus 71 etiology was detected. Whether or not the Indian strain of enterovirus 71 is less virulent than those detected in other countries in Southeast Asia remains to be seen. Nevertheless emergence of enterovirus 71 needs to be carefully monitored in India.

1. Melnick, J. L., *Rev. Infect. Dis.*, 1984, **6**, S387-S390.

2. Schmidt, N. J., Lennette, E. H. and Ho, H. H., *J. Infect. Dis.*, 1974, **129**, 304-309.
3. Melnick, J. L., in *Virology* (eds Fields, B. N., Knipe, D. M., Chanock, R. M., Melnick, J. L., Roizman, B. and Shope, R. E.), Raven Press, New York, 1985, pp. 739-794.
4. Chumakov, M. *et al.*, *Arch. Virol.*, 1979, **60**, 329-340.
5. Ishimaru, Y., Nakano, S., Yamaoka, K. and Takami, S., *Arch. Dis. Child*, 1980, **55**, 583-588.
6. Tagaya, I., Takayama, R. and Hagiwara, A., *Jpn. J. Med. Sci. Biol.*, 1981, **34**, 191-196.
7. Nagy, G., Takatsy, S., Kukan, E., Mihaly, I. and Domok, I., *Arch. Virol.*, 1982, **71**, 217-227.
8. Gilbert, G. L., Dickson, K. E., Waters, M. J., Kennett, M. L., Land, S. A. and Sneddon, M., *Pediatr. Infect. Dis. J.*, 1988, **7**, 484-488.
9. Alexander, J. P., Baden, L., Pallansch, M. A. and Anderson, L. J., *J. Infect. Dis.*, 1994, **169**, 905-908.
10. AbuBakar, S., Chee, H. Y., Al-Kobaisi, M. F., Xiaoshan, J., Chua, K. B. and Lam, S. K., *Virus Res.*, 1999, **61**, 1-9.
11. Ho, M., Chen, E. R., Hsu, K. H., Twu, S. J., Chen, K. W., Tsai, S. F., Wang, J. R. and Shih, S. R., *N. Engl. J. Med.*, 1999, **341**, 929-935.

12. McMinn, P., Stratov, I. and Dowse, G., *Commun. Dis. Intell.*, 1999, **23**, 199.
13. Lum, L. C. et al., *J. Pediatr.*, 1998, **133**, 795–798.
14. Chan, L. G. et al., *Clin. Infect. Dis.*, 2000, **31**, 678–683.
15. Brown, B. A., Oberste, M. S., Alexander, J. P. Jr., Kenne, M. L. and Pallansch, M. A., *J. Virol.*, 1999, **73**, 9969–9975.
16. McMinn, P., Lindsay, K., Perera, D., Chan, H. M., Chan, K. P. and Cardosa, M. J., *J. Virol.*, 2001, **75**, 7732–7738.
17. Brown, B. A. and Pallansch, M. A., *Virus Res.*, 1995, **39**, 195–205.
18. Banerjee, K., Hlady, W. G., Andrus, J. K., Sarkar, S., Fitzsimmons, J. and Abeykoon, P., *Bull. WHO*, 2000, **78**, 321–329.
19. Hull, B. P. and Dowdle, W. R., *J. Infect. Dis.*, 1997, **175**, S113–S116.
20. *Polio Laboratory Manual*, Department of Vaccines and Biologicals, World Health Organization, Geneva, 2001.
21. Oberste, M. S., Maher, K., Kilpatrick, D. R., Flemister, M. R., Brown, B. A. and Pallansch, M. A., *J. Clin. Microbiol.*, 1999, **37**, 1288–1293.
22. Strimmer, K. and von Haeseler, A., *Mol. Biol. Evol.*, 1996, **13**, 964–969.
23. Ricco-Hesse, R., Pallansch, M. A., Nottay, B. K. and Kew, O. M., *Virology*, 1987, **160**, 311–322.
24. Huang, C. C., Liu, C. C., Chang, Y. C., Chen, C. Y., Wang, S. T. and Yeh, T. F., *N. Engl. J. Med.*, 1999, **341**, 936–942.

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Isolation and characterization of *Yersinia enterocolitica* from diarrhoeic human subjects and other sources

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***Yersinia enterocolitica*, an important gastrointestinal pathogen, was isolated from 3% of the 1189 stool samples collected from pediatric diarrhoeic patients and 32.9% of the 492 throat swabs collected from swine in Delhi. *Y. enterocolitica* was also isolated from groundwater, waste water and river Yamuna. In addition, *Y. intermedia* and *Y. frederiksenii* were also isolated from human stool and swine throat samples. All the *Y. enterocolitica* strains belonged to biotype 1A. This study represents first isolation of *Y. enterocolitica* from swine throat swabs, groundwater and surface water in India.**

YERSINIA enterocolitica, an emerging enteric pathogen, is associated with various clinical manifestations, ranging

from self-limited gastroenteritis to more invasive syndromes like terminal ileitis and mesenteric lymphadenitis¹. *Y. enterocolitica* is commonly transmitted to humans by contaminated food and water. Swine, being the major reservoir of *Y. enterocolitica*, represent the principal source of contamination². In swine, *Y. enterocolitica* is isolated most frequently from throat. Although prevalence of *Y. enterocolitica* in temperate areas of world is well documented, there is very little information from tropical and subtropical countries, including India². Isolation of *Y. enterocolitica* from India has been reported sporadically from stools of diarrhoeic patients^{3–7}, milk⁸, swine intestinal contents⁴ and rectal swabs⁹, pork¹⁰ and sewage effluents¹¹. However, there is paucity of comprehensive studies on the isolation of *Y. enterocolitica* from India. The present study conducted over a period of three years reports isolation of *Y. enterocolitica* from pediatric diarrhoeic patients, swine throat samples, groundwater, waste water and surface water in Delhi.

A total of 1189 stool samples were collected from diarrhoeic children attending the All India Institute of Medical Sciences and Kasturba Gandhi Hospital, Delhi. In addition, 71 stool samples were also taken from non-diarrhoeic patients. Two millilitres of the stool sample was added to 18 ml of sterilized phosphate buffered saline and refrigerated at 4°C for 2 weeks. For swine, 492 samples of throat swabs were collected from four slaughterhouses located in different parts of Delhi. Each swab was transferred to 90 ml of cold enrichment broth (phosphate buffered saline with 1% sorbitol and 0.15% bile salts) and kept at 4°C for 3 weeks. A total of 179 groundwater samples were collected from handpumps, located primarily in slum areas, from all over the Delhi. Seventy-three waste water samples were taken from small and medium size waste water drains located in various parts of the city. A total of 44 surface water samples were collected from river Yamuna from the entire stretch of river running through Delhi (19 samples) and also from upstream Delhi (15 samples) and downstream Delhi (10 samples). Fifty millilitres of water sample was put in 450 ml of cold enrichment broth and kept at 4°C for 3 weeks.

After cold enrichment, samples were streaked onto CIN (Cefsulodin–Irgasan–Novobiocin) agar (Hi Media, Mumbai) plates and incubated at 25°C for 24 h (ref. 12). The presumptive *Yersinia* isolates, which showed bull's eye colony morphology on CIN agar, were subjected to four biochemical tests, viz. urease, Kligler's iron agar, differential motility and Voges-Proskauer. The isolates conforming to the above tests were subjected to detailed biochemical characterization using 46 biochemical tests¹³. Only one isolate from each positive sample was put to detailed tests. Since serotyping of *Yersinia* is a very complex process and its antisera are not available commercially, all the clinical isolates were sent to WHO *Yersinia* Reference Center, Pasteur Institute, Paris

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