

Antigenic Diversity of Enteroviruses Associated with Nonpolio Acute Flaccid Paralysis, India, 2007–2009

Technical Appendix

This supplementary material provides additional information about the authors' work on enteroviruses associated with nonpolio acute flaccid paralysis in India.

Primers for reverse transcription PCR

Oligonucleotides specific for enterovirus (EV) A, B, C, and D species, EV71, Aichivirus/Kobuvirus, Klassevirus, parechovirus, cardiovirus, and H-cosavirus were obtained from MWG Biotech (Bangalore, India). The primers contained degenerate nucleotides at specific positions. The picornavirus sequence corresponding to viral protein (VP)3 and 2A regions (VP1-specific sequences in EV71 primers) located at the 5' and 3' ends, respectively of the VP1 gene is indicated in **boldface**. The primers contained sites for desired restriction endonucleases.

EV-B-F: 5'-TACACAAGCT TGAATTCGATATC **GYWTNGYDTC**
NGNTGCAAYG AYTTC-3' (2292-2318).

EV-B-R: 5'-CAATATCTCGAGAGATCTGGATCCTGRCAYCTRGCDATDG
TRTCRCA-3' (3501-3479).

EV-A-F: 5'-TACACAAGCTTGAATTCGATATC **CCHTGGATHA**
GYAACACWCA CT-3' (2258-2280).

EV-A-R: 5'-CAATATCTCGAGAGATCTGGATCC **GYTTDGAGAA**
RCTRACRGGG TAGT-3' (3644-3621).

EV-C-F: 5'-TACACAAGCTTGAATTCGATATC **CACDATGGTD GYDCC**
WTGGAT-3' (2265-2286).

EV-C-R: 5'-CAATATCTCGAGAGATCTGGATCC **GCTARRTGGT ARTTR**
MMRAT YTTGTA-3' (3586-3561).

EV-D-F: 5'- TACACAAGCTTGAATTCGATATC **AYGCYAARKC AAYMA**
ATGCTAAT

GTT-3' (2235-2260).

EV-D-R: 5'-CAATATCTCG AGAGATCTGG ATCACTAGRA **CATCTGATTG**
CCAGTC-3' (3453-3431).

EV71-F: 5'-TACACAAGCT TGAATTCGAT ATCGTCACHY TNGTNR**TACC**
HTGGAT-3' (2207-2229).

EV71-F1: 5'-TACACAAGCT TGAATTCGAT **ATCTGGACAC TGGCAAAGTT**
CCAGCACT-3' (2555-2580).

EV71-R1: 5'-CAATATCTCG AGAGATCTGG ATCCAA**ACTA GGTTTGCCCA**
ATCATTGT-3' (3427-3405).

Aichi-Kobu-F: 5'-TACACAAGCT TGAATTCGAT ATCK**SARCCH**
CGYACCACYT TC-3' (3076-3094).

Aichi-Kobu-R: 5'- CAATATCTCG AGAGATCTGG ATCCT**GTTGG**
GRAGKKVHWC RCCR GTGAT-3' (4266-4242).

Klassevirus-F: 5'-TACACAAGCT TGAATTCGAT ATCCT**TCTGA**
WKSTRWCYYC ACCACTG-3' (2874-2894).

Klassevirus-R: 5'-CAATATCTCG AGAGATCTGG ATCCAT**CCAR**
AGWTARTAGA RWGCAA-3' (4086-4065).

Parecho-F: 5'- TACACAAGCT TGAATTCGAT ATCAAT**GCHA**
THTACACHAT HTGTGA-3'(2086-2108)

Parecho-R: 5'- CAATATCTCG AGAGATCTGG ATCCAT**CCAC**
TCYTCMACMA YDAY CCARTCTG-3' (3221-3194)

Cardiovirus-VP1-F: 5'-TACACAAGCT TGAATTCGAT ATCAC**WCTT**
GGTTTCDGGH GG-3' (2696-2713).

Cardiovirus-VP1-R: 5'-CAATATCTCG AGAGATCTGG ATCCTCGCCCA
TRCASACRAG RA-3' (3739-3721).

H-Cosavirus-VP1-F: 5'-TACACAAGCT TGAATTCGAT ATCCAYWCVN
MKDBYDVHTT CTTCT-3' (2965-2986)

H-Cosavirus-VP1-R: 5'-CAATATCTCG AGAGATCTGG
ATCCTARTCNGGRWARCCATCAA-3' (3551-3532).

The primers were constituted into 4 sets. Set 1 contained EV-B and C-specific primers, set 2 contained those specific for EV-A, EV71 and EV-D and sets 3 and 4 consisted of primers specific for Aichi/Kobu and Klasseviruses, and cardio and H-cosaviruses, respectively.

Sequenced samples

Strains from independent nonpolio acute flaccid paralysis cases = 526

Additional strains from 41 mixed infections = 45

(37 cases with 2 strains and 4 with 3 strains)

Isolates from second specimens = 43

Strains from RD-negative samples = 4

Not sequenced = 6

Total 624

Table 1. State and yearly distribution of enterovirus serotypes associated with acute flaccid paralysis (includes isolates from mixed infections and second specimens)

S no.	Serotype	2007			2008			2009			No.*
		Karnataka	Kerala	Uttar Pradesh	Karnataka	Kerala	Uttar Pradesh	Karnataka	Kerala	Uttar Pradesh	
1	CV-A2	1	-	-	-	-	-	-	-	3	4
2	CV-A3	-	-	-	-	-	-	-	-	2	2
3	CV-A4	3	-	-	1	1	2	1	-	2	10 (3)
4	CV-A5	-	-	-	-	-	-	-	-	1	1
5	CV-A6	-	-	-	-	1	2	-	-	-	3
6	CV-A7	-	-	-	3	-	-	-	-	-	3 [1]
7	CV-A8	-	-	2	1	-	-	-	-	-	3 (1)
8	CV-A9	-	-	2	-	-	2	1	-	1	6
9	CV-A10	-	-	1	-	-	-	-	-	1	2
10	CV-A14	1	-	-	-	-	3	-	2	3	9
11	CV-A16	-	-	-	-	2	1	-	-	-	3
12	CV-A17	-	-	-	1	-	-	-	-	-	1
13	CV-A21	-	-	-	-	-	1	-	-	-	1
14	CV-B1	9	-	2	2	1	3	3	-	1	21 [4]
15	CV-B2	6	-	-	-	-	3	1	1	4	15 [3]
16	CV-B3	-	1	-	-	1	3	1	-	2	8
17	CV-B4	3	-	-	2	2	2	2	-	2	13 [1]
18	CV-B5	5	-	3	2	-	11	4	-	8	33 [4]
19	CV-B6	-	-	3	7	-	2	1	-	3	16 [1]
20	E-1	-	-	3	3	-	6	1	-	-	13 [1]
21	E-2	-	-	2	1	-	4	-	-	2	9
22	E-3	3	-	-	-	-	1	3	-	2	9 [1]
23	E-4	-	-	-	-	4	1	-	-	-	5
24	E-5	-	-	1	1	-	2	-	-	2	6
25	E-6	-	1	4	1	1	5	3	-	5	20
26	E-7	3	5	1	-	3	5	1	1	4	23 [1]
27	E-9	-	-	-	1	-	-	1	-	-	2
28	E-11	1	-	-	8	-	3	5	-	10	27 [1]
29	E-12	5	-	-	-	-	-	-	-	2	7 [2]
30	E-13	7	4	1	5	2	8	4	1	13	45 [4]
31	E-14	1	2	1	6	2	8	3	-	1	24 [4]
32	E-15	1	-	-	3	-	-	-	-	-	4 [1]
33	E-17	-	1	-	-	1	-	2	-	1	5
34	E-18	-	-	-	-	-	2	2	-	-	4
35	E-19	3	-	-	2	1	6	1	-	11	24 [1]
36	E-20	-	-	-	4	-	6	-	-	3	13 [1]
37	E-21	1	-	1	-	-	4	1	-	-	7
38	E-24	-	-	5	-	-	3	3	-	-	11 [1]
39	E-25	-	1	1	4	-	7	-	-	2	15
40	E-26	-	-	-	-	-	1	-	-	-	1
41	E-27	-	-	-	-	-	1	-	-	-	1
42	E-29	4	-	7	4	-	3	-	-	-	18 [2]
43	E-30	3	-	3	3	1	-	-	-	8	18 [2]
44	E-31	-	-	-	-	-	-	1	-	1	2
45	E-32	-	-	3	2	-	1	1	-	1	8
46	E-33	4	2	1	3	1	2	-	-	9	22 [3]
47	EV-69	-	-	-	1	-	4	1	-	6	12
48	EV-71	-	7	1	1	11	12	9	1	8	50 [1]
49	EV-73	-	-	-	1	-	1	-	-	-	2
50	EV-74	-	-	1	-	-	2	-	-	-	3
51	EV-75	1	-	5	1	-	2	1	1	2	13 [1]
52	EV-76	-	-	-	-	-	-	-	-	1	1
53	EV-78	-	-	-	-	-	3	-	-	1	4
54	EV-79	-	-	1	1	-	-	1	-	-	3
55	EV-80	2	-	1	1	-	-	-	1	-	5 [1]
56	EV-81	-	-	-	-	-	1	-	-	-	1
57	EV-83	-	-	2	-	-	3	-	-	1	6 [1]
58	EV-84	-	-	-	1	-	3	-	-	-	4
59	EV-86	-	-	-	1	-	-	-	-	-	1
60	EV-88	-	-	-	1	-	-	-	-	-	1
61	EV-89	-	-	-	-	-	1	-	-	-	1
62	EV-90	-	2	-	-	-	-	-	-	1	3
63	EV-93	-	-	-	-	-	3	-	-	1	4
64	EV-97	1	-	3	-	-	1	2	-	1	8
65	EV-100	-	-	2	-	-	-	-	-	2	4

S no.	Serotype	2007			2008			2009			No.*
		Karnataka	Kerala	Uttar Pradesh	Karnataka	Kerala	Uttar Pradesh	Karnataka	Kerala	Uttar Pradesh	
66	EV-107	–	–	–	–	–	–	–	–	1	1
Total		68	26	63	79	35	150	60	8	135	624

*Numbers in parentheses represent isolates identified from cell culture negative nonpolio acute febrile paralysis samples by nucleotide sequencing. Numbers in brackets represent isolates from second specimens RD-positive cases for which viral protein 1 sequences were determined.

Table 2. Enterovirus species, serotypes and isolates associated with acute flaccid paralysis for which nucleotide sequence and the nucleotide and amino acid identities among the isolates of each serotype were determined

S. no.	Serotype	Species	State			No. isolates	Identity, %		Isolates
			KA	UP	KE		nt	aa	
1	CV-A2	HEV-A	1	3	–	4	80–81	94–95	N-115,845, 850A, 859
2	CV-A3	HEV-A	–	2	–	2	76	91	N-982, 984
3	CV-A4	HEV-A	5	4	1	10	85–87	89–97	N-48, 52, 56 , 183B, 253,357, 391, 928, 980,1062
4	CV-A5	HEV-A	–	1	–	1	73	90	N-864
5	CV-A6	HEV-A	–	2	1	3	78–82	87–96	N-258, 313, 384
6	CV-A7	HEV-A	3	–	–	3	81	93–95	N-132, 133 , 183C
7	CV-A8	HEV-A	1	2	–	3	85	97–98	N-650 , 662, 672
8	CV-A9	HEV-B	1	5	–	6	78–79	91–95	N-451A,452,656,683,828,924
9	CV-A10	HEV-A	–	2	–	2	78–79	94–95	N-734, 992
10	CV-A14	HEV-A	1	6	2	9	80–84	89–97	N-92,470,471, 473, 510, 576, 994, 1047, 1055
11	CV-A16	HEV-A	–	1	2	3	74–76	87–90	N-442, 450B, 498
12	CV-A17	HEV-C	1	–	–	1	75	91	N-142
13	CV-A21	HEV-C	–	1	–	1	75	93	N-265A
14	CV-B1	HEV-B	14	6	1	21	72–98	88–96	N-64, 65 , 66, 67 , 74, 75 , 77, 78 , 90, 181, 209, 245, 251, 385, 408, 671, 705, 850B, 907, 911, 916
15	CV-B2	HEV-B	7	7	1	15	82–84	96–98	N-1, 2 , 68, 69 , 81, 82 , 375, 430, 454, 757, 791, 797, 816, 1002, 1054
16	CV-B3	HEV-B	1	5	2	8	78–79	95–97	N-249, 338, 359, 378, 379, 773, 800, 823
17	CV-B4	HEV-B	7	4	2	13	80–82	97–98	N-20, 118, 119 , 183A, 186, 290A, 372, 497, 504,802B, 917, 941, 966A
18	CV-B5	HEV-B	11	22	–	33	81–83	96–99	N-10, 13, 14 , 72, 73 , 167, 168 , 232, 252, 254, 257, 259, 277B, 278B , 353, 355B, 424, 462, 592, 602,719, 749, 832, 863, 901B, 904A, 922, 972, 973, 1013, 1019B, 1031,1061
19	CV-B6	HEV-B	8	8	–	16	75–84	84–96	N-165, 179, 180 ,184, 187, 199, 227, 360A, 361 , 615, 695, 711, 756, 978, 990A,1000B
20	E-1	HEV-B	4	9	–	13	75–82	92–97	N-136, 196, 272, 367, 368 , 400, 407, 482, 483, 658, 690, 733, 772
21	E-2	HEV-B	1	8	–	9	79–81	91–94	N- 220, 285, 406, 440, 441,668,742, 947, 1059A
22	E-3	HEV-B	6	3	–	9	79–81	94–97	N-116, 121, 122 , 405, 755, 763, 939, 1010, 1015
23	E-4	HEV-B	–	1	4	5	78–81	93–97	N-364, 510, 515, 517, 518
24	E-5	HEV-B	1	5	–	6	75–80	88–95	N-173, 468, 487, 713, 802A, 844
25	E-6	HEV-B	4	14	2	20	74–78	91–95	N-236, 284, 288, 333, 365, 394B,416, 587, 664, 700, 727, 909, 931, 932, 961,966B, 975A, 961,985, 1007
26	E-7	HEV-B	4	10	9	23	75–84	87–94	N-15, 16 ,89,240,276,290A,301,311,340,342,348, 352,381,392,394,572,699,750,783,956,957,959, 1021
27	E-9	HEV-B	2	–	–	2	78–79	89–97	N-460, 933
28	E-11	HEV-B	14	13	–	27	77–81	91–98	N-104, 194, 197, 202, 214, 216, 222, 228, 229 , 265B,281,409,908A,910,912,915,919,968,976,99 1,998A,999, 1009, 1028,1032,1046A,1064
29	E-12	HEV-B	5	2	–	7	78–82	96–98	N-79, 80 , 88, 100, 101 , 801, 1072
30	E-13	HEV-B	16	22	7	45	75–79	84–94	N- 4,5,7, 8 ,9,102,103,139,190,213,225, 226 ,309, 310 , 316,327,331,349,355A,387,425,459,469,486,503 ,577,738,764,779,788,794,849,911A,914,953,97 6B,979,981,983,986,997,1003,1030, 1012,1074
31	E-14	HEV-B	10	10	4	24	75–80	83–95	N-91, 125, 126 , 129, 130 , 201, 218, 230, 231 , 279, 280 ,320,323,325,337,413,437,500,575,613,925, 940,941A,1060
32	E-15	HEV-B	4	–	–	4	76–90	87–88	N-109, 131, 161, 162
33	E-17	HEV-B	2	1	2	5	80–82	93–95	N-324, 382, 748, 936, 1017
34	E-18	HEV-B	2	2	–	4	78–80	93–95	N-242, 291, 766, 904B
35	E-19	HEV-B	6	17	1	24	77–78	88–99	N-

S. no.	Serotype	Species	State			No. isolates	Identity, %		Isolates
			KA	UP	KE		nt	aa	
									86, 87 ,96,182,205,261,417,426,429,431,432,513,744,795,949,950,964,975A,988,1004B,1008,1014,1025,1056
36	E-20	HEV-B	4	9	–	13	77–80	91–97	N-144, 176, 178, 263, 292, 297, 369, 370 , 411, 456, 948, 1046B, 1061
37	E-21	HEV-B	2	5	–	7	77–80	91–96	N-123, 410, 412, 422, 485, 726, 937
38	E-24	HEV-B	3	8	–	11	77–79	87–97	N- 282, 283 , 458, 661, 712, 714, 731, 736, 754, 935, 943
39	E-25	HEV-B	4	10	1	15	77–80	89–94	N-146, 156, 171, 273, 315,326, 356, 401, 404, 418, 438, 491, 706, 831, 862
40	E-26	HEV-B	–	1	–	1	78	88	268
41	E-27	HEV-B	–	1	–	1	77	88	256
42	E-29	HEV-B	8	10	–	18	78–80	93–96	N-70, 71 , 98, 99 , 153, 155, 193, 305, 398, 414, 435, 591, 655, 674, 678, 693A, 701, 728
43	E-30	HEV-B	6	11	1	18	75–81	88–98	N-85, 107, 108 , 148, 188, 189 , 505,584, 614, 665, 847, 944, 958, 995, 1000A, 1001, 1004A, 1019
44	E-31	HEV-B	1	1	–	2	80–81	96–98	N-761, 829
45	E-32	HEV-B	3	5	–	8	77–80	91–93	N-224, 443, 465, 722, 723, 737, 900, 990A
46	E-33	HEV-B	7	12	3	22	77–79	93–97	N-21, 22 ,105, 106 ,138,151, 152A ,329,346,419,420,519,730,873,977,998,1029A,1034,1058,1069,1059B,1071
47	EV-69	HEV-B	2	10	–	12	76–80	88–94	N-152B, 318, 434B, 439, , 453, 850C, 909A, 966C, 970, 974, 985A, 1050
48	EV-71	HEV-A	10	21	19	50	75–81	88–96	N-384,386,389, 390 ,391B,427,444,445,446,447,449,450,451,471,474,475,480,490,493,494,498,502,505A,506,507,514,516,571,574,578,579,651,789,845,894,899,901A, 903, 905, 906, 910A, 918, 942, 955, 963, 975, 987, 993, 998, 1024
49	EV-73	HEV-B	1	1	–	2	75	88–96	N-150, 450A
50	EV-74	HEV-B	–	3	–	3	79	90–95	N-433, 434A , 589
51	EV-75	HEV-B	3	9	1	13	84	92–100	N-83, 211, 247, 248 , 675, 684, 688, 694, 741, 784, 938, 951, 1043
52	EV-76	HEV-A	4	1	–	5			N-894, 895, 899, 905,1042
53	EV-78	HEV-B	–	4	–	4	79–83	94–97	N-303, 455, 457, 1039
54	EV-79	HEV-B	2	1	–	3	79–82	96–97	N-169, 696, 931
55	EV-80	HEV-B	3	1	1	5	75–79	89–96	N-94, 95 , 127, 660, 790
56	EV-81	HEV-B	–	1	–	1	79	95	N-428
57	EV-83	HEV-B	–	6	–	6	79–80	90–95	N-277, 278 , 489, 685, 698, 833
58	EV-84	HEV-B	1	3	–	4	81	96	N-246, 472, 476, 481
59	EV-86	HEV-B	1	–	–	1	89	98	N-160
60	EV-88	HEV-B	1	–	–	1	83	94	N-206
61	EV-89	HEV-A	–	1	–	1			N-286
62	EV-90	HEV-A	–	1	2	3			N-332, 341, 1052
63	EV-93	HEV-B	–	4	–	4	89–92	97–99	N-289, 423, 461, 1073
64	EV-97	HEV-B	3	5	–	8	90–97	93–98	N-6, 360B, 659, 693B, 729, 774, 804, 903
65	EV-100	HEV-B	–	4	–	4	82–85	93–96	N-653, 667, 821, 837
66	EV-107	HEV-B	–	1	–	1	81	93	N-842

KA, Karnataka; UP, Uttar Pradesh; KE, Kerala; HEV, human enterovirus. Independent strains from RD-positive cases 577, and isolates from second collection specimens 43 and strains from RD-negative cases 4. Numbers in **boldface** represent isolates from second specimens and those in **bold italics** represent isolates from cell culture–negative samples from children with nonpolio acute paralytic.

Table 3. Enterovirus serotypes detected in mixed infections in children with AFP*

1. N845-EV21 & N845A-EV71
2. N391-Cox A4 & N391A-EV71
3. N451A-CoxA9 & N451-EV71
4. N471-CoxA14 & N471A-EV71
5. N498A-CoxA16 & N498-EV71
6. N505- Ev30 & N505A- EV71
7. N901B-CoB5 & N901A-EV71
8. N998A-EV11 & N998B-EV71
9. N490B-EV30 & N490A-EV71
10. N903A-EV97 & N903- EV71
11. N384-A6 & N384A-EV71
12. N910-EV11 & N910A-EV71
13. N975A-EV6 & N975B-EV19 & N975C-EV71
14. N277B-CoxB5 & N277A-EV83
15. N355B-CoxB5 & N355A-EV13
16. N904A-CoxB5 & N904B-EV18
17. N1019B-CoxB5 & N1019A-EV30
18. N941-CoxB4 & N941A-EV14
29. N290A-CoxB4 & N290B-EV7
20. N802B-CoxB4 & N802A-EV5
21. N966A-CoxB4 & N966B-EV6 & N966C-EV69
22. N183B-CoxA4 & N183A-CoxB4 & N183C-CoxA7
23. N360A-CoxB6 & N360B-EV97
24. N990A-CoxB6 & N990B-EV32
25. N1000B-CoxB6 & N1000A-EV30
26. N911- CoxB1 & N911A-EV13
27. N850A-CoxA2 & N850-B-CoxB1 & N850C-EV69
28. N510- EV4 & N510A-CoxA14
39. N1059A-EV2 & N1059B-EV33
30. N909- EV6 & N909A-EV69
31. N985- EV6 & N985A-EV69
32. N931-EV6 & N931A-EV69
33. N394B-EV6 & N394-EV7
34. N434A-EV9 & N434B-EV69
35. N1046A-EV11 & N1046B-EV20
36. N976A-EV11 & N976B-EV13
37. N1004B-EV19 & N1004A-EV30
38. N693A-EV29 & N693B-EV97
49. N152A-EV33 & N152B-EV69
40. N450B-CoxA16 & N450A-EV73
41. N265A-CoxA21 & N265B-EV11

*AFP, acute flaccid paralysis.