BRIEF REPORT

Genetic characterization of a novel recombinant echovirus 30 strain causing a regional epidemic of aseptic meningitis in Hokkaido, Japan, 2017

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Abstract

A regional epidemic of aseptic meningitis caused by echovirus 30 (E30) occurred in Hokkaido, Japan, during the period of August-December 2017. To investigate their phylogenetic relationship to other human enteroviruses, we determined the complete genomic nucleotide sequences of isolates from this outbreak. Phylogenetic analysis of the viral capsid protein 1 gene showed that the strains were most closely related to E30 strains detected in Germany, France, and Russia in 2013. In contrast, the region encoding the viral protease and the RNA-dependent RNA polymerase had a close phylogenetic relationship to non-E30 enteroviruses detected in the United Kingdom and Switzerland in 2015-2017, suggesting that a recombination event had occurred.

Keywords Echovirus 30 · Aseptic meningitis · Outbreak · Genetic characterization · Genome recombination

Echovirus 30 (E30) is one of the serotypes belonging to the species *Enterovirus B* [1] and is a major pathogen causing aseptic meningitis (AM) in both children and adults. Large epidemics caused by E30 have been frequently reported globally [2]. We experienced a regional epidemic of AM caused by E30 in Hokkaido, Japan, during the period of August-December 2017. Details of the epidemic and the clinical features were presented previously [3]. In this study, we determined the complete nucleotide sequences of viruses from the outbreak to determine their molecular features and

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their phylogenetic relationship to other strains of enterovirus B (EV-B). Virus isolation, RNA extraction, and sequencing were performed as described earlier [4]. Genome amplification and the nucleotide sequencing were carried out using specific primers (Supplementary Table S1). We determined the complete nucleotide sequences of four isolates, named E30/Hokkaido.JPN/21208 (Hokkaido21208), 21214, 21246, and 21326, that were collected on August 31, September 2 and 18, and November 24, 2017, and submitted them to the GenBank database under the accession numbers LC416533 to LC416536, respectively.

To investigate the genetic relationships of these isolates to globally circulating strains, phylogenetic analysis based on the viral capsid protein 1 (VP1) gene, the viral protease gene, and the RNA-dependent RNA polymerase (3CD) gene were carried out. Phylogenetic analysis based on the VP1 gene (876 bases) was performed in comparison with 248 E30 strains isolated in 33 countries during 1957-2017, and analysis based on the 3CD gene (1938 bases) was performed with 243 EV-B strains isolated in 33 countries during 1948-2017 (Supplementary Tables S2 and S3). With respect to choosing the strains, we took into account the genetic classifications of the strains that had been evaluated [2, 4–7]. Phylogenetic trees were constructed using Molecular Evolutionary Genetic Analysis software (MEGA v.5) [8], using
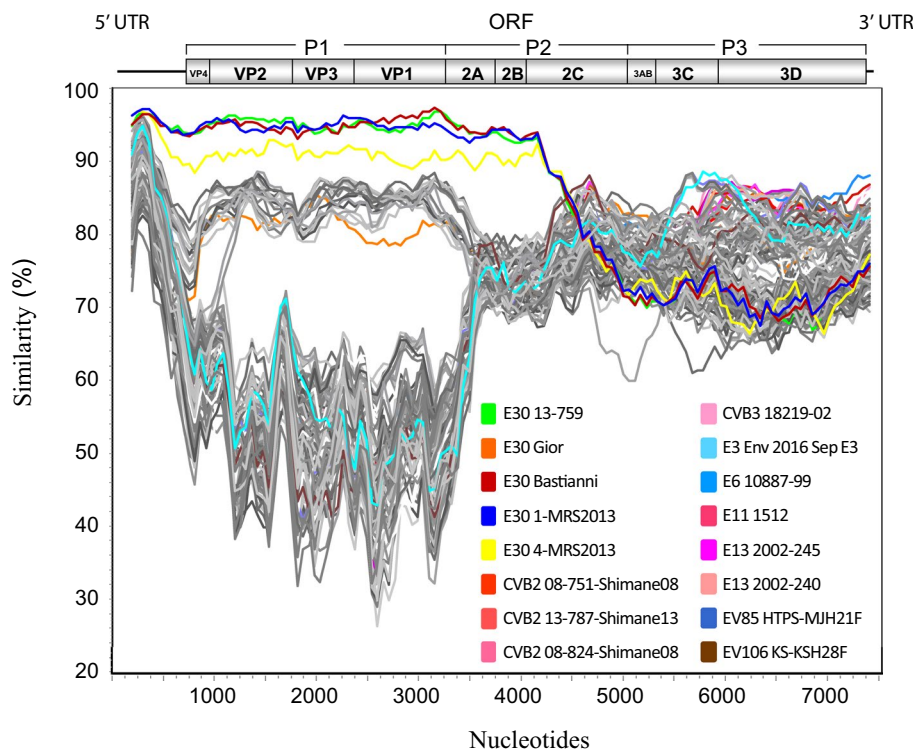
the maximum-likelihood method based on the general time-reversible model. The genetic lineages were confirmed by bootstrap values using 1000 pseudoreplicates [9]. Furthermore, the nucleotide sequences were aligned with those of 243 strains (Supplementary Table S3) of EV-B using MEGA

Table 1 Nucleotide and amino acid sequence identity between strain Hokkaido21208 and strain 13-759

Genomic region	No. of Nucleotide/ Amino acid (base/residue)	Each Identity (%)
5'UTR	742 / -	96.0 / -
P1		
VP4	210 /70	94.3 /95.7
VP2	780 /260	96.2 /99.6
VP3	714 /238	95.8 /99.6
VP1	876 /292	96.1 /99.3
P2		
2A	450 /150	95.1 /98.0
2B	297 /99	92.3 /96.0
2C	987 /329	84.0 /98.2
P3		
3A	267 /89	78.7 /96.6
3B	66 /22	71.2 /90.9
3C	549 /183	79.2 /96.7
3D	1389 /462	80.3 /97.0
3'UTR	101/ -	79.2 / -

Black arrow indicates the predicted recombination breakpoint.

v.5. The gap-opening and extension penalties were set to 15 and 6.66, respectively, and the alignments were corrected manually to match the open reading frame (ORF). In order to identify potential recombination sites in the genome, a similarity plot analysis was performed using Simplot software version 3.5.1 [10] with a sliding window of 400 nucleotides moving in steps of 50 nucleotides.

The genomes of four E30 strains from Hokkaido consisted of 7428 nucleotides including a 5' untranslated region (UTR) of 742 nucleotides followed by an ORF encoding a polypeptide of 2194 amino acids and a 3' UTR of 101 nucleotides. The nucleotide and amino acid sequences of these strains were highly conserved (99.6-9% and 99.8-9% identity), suggesting that a single lineage of E30 had spread. The sequence of the 5' UTR and the P1 genomic region of the strain Hokkaido21208 showed the highest identity to those of the strain 13-759, which was isolated in Germany in 2013 [11] (Table 1). While a high degree of nucleotide sequence similarity was also found in the 2A and the 2B genes, the sequence identity values from the 2C gene to the 3' UTR were considerably lower (ranging from 71.2% to 84.0%). However, despite this divergence, the amino acid identity values from the P1 region to the P3 region ranged from 90.9% to 99.6% (Table 1).

The result of similarity plot analysis correlated with the above results (Fig. 1). From the 5' UTR to the 2B gene, the

Fig. 1 Similarity plot analysis comparing strain Hokkaido21208 with EV-B strains. The nucleotide sequence of strain Hokkaido21208 was compared with those of 223 strains of EV-B (Supplementary Table S3), using Simplot software version 3.5.1 with a sliding window of 400 nucleotides moving in steps of 50 nucleotides. The strains described in this article are indicated by colored lines, and other strains are indicated by gray lines

strain Hokkaido21208 was most closely related to the strain 13-759. However, the similarity of the 2C gene dropped abruptly to the same level as with other serotypes of EV-B. By contrast, the genomic region between the 2C gene and the 3' UTR of the strain Hokkaido21208 did not show significant similarity to any of the strains compared, suggesting that a recombination event had occurred at the border between the 2B and 2C genes. This breakpoint has been previously identified as a recombination hot spot [12].

A previous study indicated that the VP1 genes of E30 detected in Japan before 1998 were divided into six genogroups [5]. These genogroups correspond to the lineages E30_d, e, and g based on an advanced report by Bailly et al. [6]. In this study, our analysis indicated that the Hokkaido strains belong to the lineage E30_f (Fig. 2A), forming a novel cluster with Japanese strains detected in Fukushima prefecture, and with German, French, and Russian strains detected during 2008-2014. In contrast, several Japanese strains detected during 2008-2014 in Toyama, Osaka, Oita, and Okinawa prefectures, located mainly in western Japan, were classified in lineage E30_b (Fig. 2A). These data suggest that the Hokkaido strains might have originated overseas, in particular, Europe. E30 strains have been detected almost every year in Japan [13]. However, due to insufficient genetic information of these isolates, the detailed phylogenetic and topological relationships among Japanese strains and the time point of importation of lineage E30_f could not be clarified.
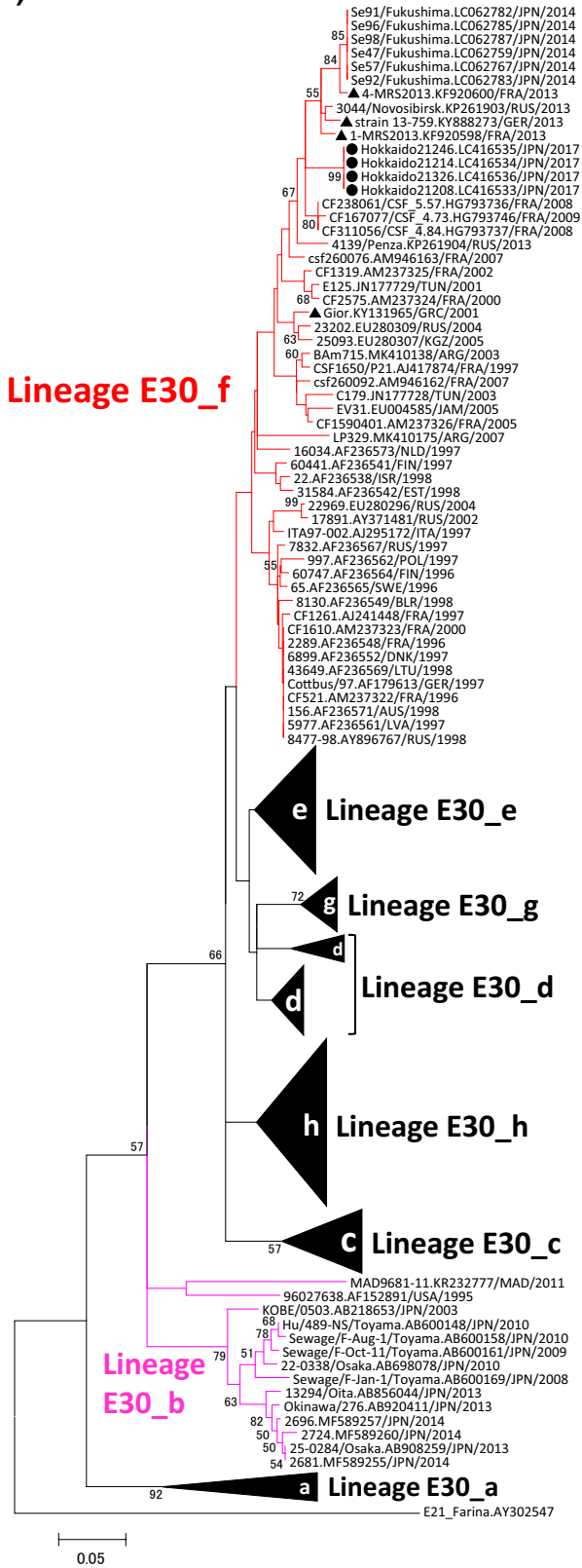
Amino acid substitutions in VP1 of the Hokkaido strains were found in at least 20 positions in comparison with the strain 25-0284/osaka.AB908259/JPN/2013, which belongs to lineage E30_b (Table 2). Among these sites, 13 substitutions (56Y/F, 87E/D, 120I/V, 145V/I, 156R/K, 157S/G, 234N/S, 247K/R, 269V/I, 274E/D, 284S/T, 285T/N, and 289L/M) showed divergence from these lineages. Codons 87 and 156, which comprise a portion of the BC loop and the EF loop, respectively, have been predicted not only to be exposed on the surface but also to represent putative positive selection sites [6, 14]. Besides, several substitutions are in the C-terminal region of VP1 (amino acids 260-292), which has been recognized as one of the main antigenic regions of enteroviruses [15–17]. The emergence of an epidemic would implicitly indicate the lack of herd immunity in the community. Furthermore, the novel strains, which have different antigenic properties, even within the same serotype, could have escaped the host's immune system. Earlier studies have demonstrated that E30 is antigenically heterogeneous and divided into three major groups [18, 19]. The VP1 nucleotide sequences of these groups are classified into the lineage E30_a or c, while the differences in antigenic properties do not correlate with the comprehensive classification [6]. Under these circumstances, it is important to explore whether the strains are pathogenic and able to spread beyond a regional scale in the near future. However, the strain-specific features that might be involved in disease are still not well understood [11, 20], and more-detailed study is needed to evaluate the characteristics of each lineage and to understand the potential endemic risk and the dynamics of virus transmission.

The 3CD genes of EV-B are divided phylogenetically into three groups (1, 2, and 3) (Fig. 2B) [4, 7]. Our findings indicate that the Hokkaido strains belong to group 3 and form a cluster with coxsackievirus type B2 (CVB2), CVB3, CVB4, CVB5, E3, E6, E11, E13, enteroviruses 85, and B106 strains detected in European and Asian countries during the last 20 years. Among these strains, CVB4, CVB5, and E3 strains detected in the United Kingdom and Switzerland during 2015-2017 occupied the nearest branch (Fig. 2B). No other E30 strain except for the Hokkaido strains was found in this cluster. In contrast, the E30 strain 13-759, whose VP1 gene showed a high degree of similarity to those of the Hokkaido strains, was classified into group 2. Group 2 included several EV-B strains detected intermittently in Europe, suggesting that this genome has been maintained there since the previous century (Fig. 2B) [6]. In addition to the data for the VP1 genes described above, chronological and phylogenetic information about the 3CD genes strongly suggest that the Hokkaido E30 strains originated in Europe.

The emergence of E30 has been found to exhibit a cyclic pattern with dominance and disappearance of individual recombinants over a period of years [21–27]. The turnover period for such recombinants has been calculated to be from 1.8 to 5 years [28, 29]. Therefore, long-term surveillance based on detection of virus in clinical specimens and environmental materials would contribute to our understanding trends and epidemiological patterns [30–34]. Furthermore, investigation of the antigenic properties of circulating viruses is fundamental to understand their pathogenic features and, ultimately, to promote measures that are important for preventive public health.
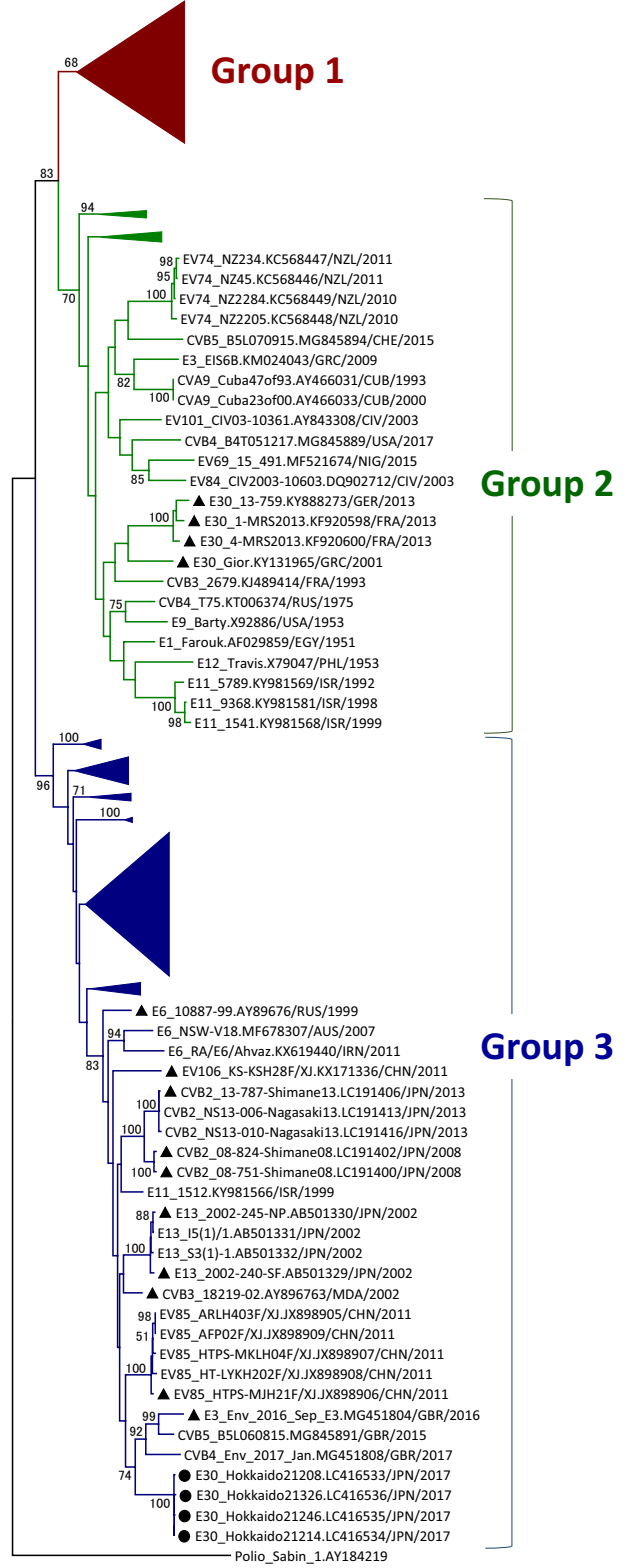
(A)

Se91/Fukushima.LC062782/JPN/2014
Se96/Fukushima.LC062785/JPN/2014
Se98/Fukushima.LC062787/JPN/2014
Se47/Fukushima.LC062759/JPN/2014
Se57/Fukushima.LC062767/JPN/2014
Se92/Fukushima.LC062783/JPN/2014
▲ 4-MRS2013.KF920600/FRA/2013
3044/Novosibirsk.KP261903/RUS/2013
▲ strain 13-759.KY888273/GER/2013
▲ 1-MRS2013.KF920598/FRA/2013
● Hokkaido21246.LC416535/JPN/2017
● Hokkaido21214.LC416534/JPN/2017
● Hokkaido21326.LC416536/JPN/2017
● Hokkaido21208.LC416533/JPN/2017
CF238061/CSF_5.57.HG793736/FRA/2008
CF167077/CSF_4.73.HG793746/FRA/2009
CF311056/CSF_4.84.HG793737/FRA/2008
4139/Penza.KP261904/RUS/2013
csf260076.AM946163/FRA/2007
CF1319.AM237325/FRA/2002
E125.JN177729/TUN/2001
CF2575.AM237324/FRA/2000
▲ Gior.KY131965/GRC/2001
23202.EU280309/RUS/2004
25093.EU280307/KGZ/2005
BAm715.MK410138/ARG/2003
CSF1650/P21.AJ417874/FRA/1997
csf260092.AM946162/FRA/2007
C179.JN177728/TUN/2003
EV31.EU004585/JAM/2005
CF1590401.AM237326/FRA/2005
LP329.MK410175/ARG/2007
16034.AF236573/NLD/1997
60441.AF236541/FIN/1997
22.AF236538/ISR/1998
31584.AF236542/EST/1998
22969.EU280296/RUS/2004
17891.AY371481/RUS/2002
ITA97-002.AJ295172/ITA/1997
7832.AF236567/RUS/1997
997.AF236562/POL/1997
60747.AF236564/FIN/1996
65.AF236565/SWE/1996
8130.AF236549/BLR/1998
CF1261.AJ241448/FRA/1997
CF1610.AM237323/FRA/2000
2289.AF236548/FRA/1996
6899.AF236552/DNK/1997
43649.AF236569/LTU/1998
Cottbus/97.AF179613/GER/1997
CF521.AM237322/FRA/1996
156.AF236571/AUS/1998
5977.AF236561/LVA/1997
8477-98.AY896767/RUS/1998

Lineage E30_f

85
84
55
67
99
80
68
63
60
99
55

e Lineage E30_e

g Lineage E30_g 72
d
d **Lineage E30_d**
66

h Lineage E30_h

57
c Lineage E30_c 57

MAD9681-11.KR232777/MAD/2011
96027638.AF152891/USA/1995
KOBE/0503.AB218653/JPN/2003
Hu/489-NS/Toyama.AB600148/JPN/2010
Sewage/F-Aug-1/Toyama.AB600158/JPN/2010
Sewage/F-Oct-11/Toyama.AB600161/JPN/2009
22-0338/Osaka.AB698078/JPN/2010
Sewage/F-Jan-1/Toyama.AB600169/JPN/2008
13294/Oita.AB856044/JPN/2013
Okinawa/276.AB920411/JPN/2013
2696.MF589257/JPN/2014
2724.MF589260/JPN/2014
25-0284/Osaka.AB908259/JPN/2013
2681.MF589255/JPN/2014

Lineage E30_b

68
78
79
51
63
82
50
50
54

a Lineage E30_a 92

E21_Farina.AY302547

0.05

(B)

68 ◄ **Group 1**

83
94

98 EV74_NZ234.KC568447/NZL/2011
95 EV74_NZ45.KC568446/NZL/2011
100 EV74_NZ2284.KC568449/NZL/2010
EV74_NZ2205.KC568448/NZL/2010
CVB5_B5L070915.MG845894/CHE/2015
E3_EIS6B.KM024043/GRC/2009
82 CVA9_Cuba47of93.AY466031/CUB/1993
100 CVA9_Cuba23of00.AY466033/CUB/2000
EV101_CIV03-10361.AY843308/CIV/2003
CVB4_B4T051217.MG845889/USA/2017
EV69_15_491.MF521674/NIG/2015
85 EV84_CIV2003-10603.DQ902712/CIV/2003
▲ E30_13-759.KY888273/GER/2013
100 ▲ E30_1-MRS2013.KF920598/FRA/2013
▲ E30_4-MRS2013.KF920600/FRA/2013
▲ E30_Gior.KY131965/GRC/2001
CVB3_2679.KJ489414/FRA/1993
75 CVB4_T75.KT006374/RUS/1975
E9_Barty.X92886/USA/1953
E1_Farouk.AF029859/EGY/1951
E12_Travis.X79047/PHL/1953
E11_5789.KY981569/ISR/1992
100 E11_9368.KY981581/ISR/1998
98 E11_1541.KY981568/ISR/1999

Group 2

70
96
100
71
100

Group 3

▲ E6_10887-99.AY89676/RUS/1999
94 E6_NSW-V18.MF678307/AUS/2007
E6_RA/E6/Ahvaz.KX619440/IRN/2011
83 ▲ EV106_KS-KSH28F/XJ.KX171336/CHN/2011
▲ CVB2_13-787-Shimane13.LC191406/JPN/2013
100 CVB2_NS13-006-Nagasaki13.LC191413/JPN/2013
100 CVB2_NS13-010-Nagasaki13.LC191416/JPN/2013
▲ CVB2_08-824-Shimane08.LC191402/JPN/2008
100 ▲ CVB2_08-751-Shimane08.LC191400/JPN/2008
E11_1512.KY981566/ISR/1999
88 ▲ E13_2002-245-NP.AB501330/JPN/2002
E13_I5(1)/1.AB501331/JPN/2002
100 E13_S3(1)-1.AB501332/JPN/2002
▲ E13_2002-240-SF.AB501329/JPN/2002
▲ CVB3_18219-02.AY896763/MDA/2002
98 EV85_ARLH403F/XJ.JX898905/CHN/2011
51 EV85_AFP02F/XJ.JX898909/CHN/2011
EV85_HTPS-MKLH04F/XJ.JX898907/CHN/2011
100 EV85_HT-LYKH202F/XJ.JX898908/CHN/2011
▲ EV85_HTPS-MJH21F/XJ.JX898906/CHN/2011
99 ▲ E3_Env_2016_Sep_E3.MG451804/GBR/2016
92 CVB5_B5L060815.MG845891/GBR/2015
CVB4_Env_2017_Jan.MG451808/GBR/2017
74 ● E30_Hokkaido21208.LC416533/JPN/2017
100 ● E30_Hokkaido21326.LC416536/JPN/2017
● E30_Hokkaido21246.LC416535/JPN/2017
● E30_Hokkaido21214.LC416534/JPN/2017

Polio_Sabin_1.AY184219

0.1

Table 2 Amino acid substitutions in VP1 of E30 strains detected in Japan after 2010

Strain	Lineage	Amino acid number																				
		56	84	87	100	120	145	156	157	204	234	247	263	269	274	277	284	285	287	288	289	290
Hokkaido strains/2017	f	Y	V	E	M	I	V	R	S	S	N	K	R	V	E	G	S	T	N	P	L	S
Se47/Fukushima/2014	f	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Se91/Fukushima/2014	f	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.
Se98/Fukushima/2014	f	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L
25-0284/Osaka/2013	b	F	A	D	.	V	I	K	G	N	S	R	K	I	D	S	T	N	G	V	M	K
22-0338/Osaka/2010	b	F	A	D	.	V	I	K	G	N	S	R	K	I	D	S	T	N	S	A	M	M
489-NS/Toyama/2010	b	F	A	D	.	V	I	K	G	.	S	R	.	I	D	F	T	N	*	*	*	*
F-Aug-1/Toyama/2010	b	F	A	D	I	V	I	K	G	N	S	R	.	I	D	S	T	N	*	*	*	*

Dots (.) indicate amino acid residues identical to those in the Hokkaido strains

Asterisks (*) indicate that there was no sequencing information

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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