Genetic diversity of the enteroviruses detected from Cerebrospinal fluid (CSF) samples of patients with suspected aseptic meningitis in northern West Bank, Palestine in 2017

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Background

Human enterovirus (HEV) is a non-enveloped RNA genus of the family *Picornaviridae* with more than 107 genotypes. HEV is divided into 4 HEV species designated HEVA to D, based on the sequences analysis of the partial or complete VP1 region of the viral genome[1]. The VP1 are immunodominant capsid proteins that contain the neutralization antigenic determinants which correlate well with serotyping and are used for HEV genotyping[2].

HEVs are associated with several diverse clinical manifestations including mild febrile illness, gastroenteritis, respiratory tract infection, neonatal sepsis-like illness and aseptic meningitis as the most frequent infections caused by HEV that occurs as sporadic and/or outbreaks of varying size. HEV-B causing aseptic meningitis include echoviruses, some enteroviruses coxsachievirus B[3-5]. Furthermore, a sever and potentially fatal conditions including encephalitis, acute flaccid paralysis, myocarditis and hand-foot-mouth disease had been reported [6-10].

In the last two decades, echovirus18 (E18) and coxsackievirus B5 (CVB5), serotypes of HEV-B, have been isolated from sporadic and outbreak cases of aseptic meningitis in China, Taiwan, Germany, Korea, Australia and Palestine. These isolates were found to be associated with various degrees of illness among newborns, infants and school-age children, ranging from asymptomatic to fatal aseptic meningitis [10-17].

The high mutation rate and the RNA recombination are responsible for genetic diversity of the enteroviruses [18].The recombination increases viral pathogenicity, eliminates lethal mutations and increases fitness of virus [18]. Of the four species, A, B, C and D, HEV-B was shown to have the highest rates of recombination particularly between members of the same species [19-20].The few studies that investigated the molecular characterization of the two HEVs (E18 and CVB5),

showed a wide range of genetic diversity in the VP1 region coding for the outer surface protein, which involved in virus–cell interactions that might explain their high endemicity and infection severity among the newborns, infants, children and adults[12-16]. The aim of the present study was to identify the most predominant enteroviruses which circulated in the northern West Bank, Palestine in 2017 using RT-PCR followed by sequencing and to investigate the genetic diversity of the sequences of the partial VP1 region.

Materials and Methods

Sample and data collection

During the period of March, 1st to October, 31th, 2017, 249 cerebrospinal fluid (CSF) specimens were collected from children admitted, with suspected aseptic meningitis, to Rafeedia governmental hospital in Nablus and Jenin governmental Hospital in Jenin and were store at -20 ^{O}C until use. All CSF samples were proven negative for classical bacterial pathogens by the hospital microbiology laboratory. Patients' demographic data and clinical history including age, sex, sign and symptoms, place of residence, date of onset of symptoms, and CSF laboratory test results were retrieved from the patients' files. The study was approved by the Ministry of Health in Palestine under reference number ATM/125/2013

Viral RNA extraction

The HEV RNA was extracted from 200 µl of CSF using a QIAamp viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions

74 # RT-PCR

75 To identify the enterovirus associated aseptic meningitis cases, the HEV RNA was amplified using
76 two primer sets targeting the 5'NCR of the HEV genome as described and modified previously[10,
77 21]. For HEV genotyping, an RT-PCR with two primer sets was used to amplify the 5' half of the
78 VP1region of the viral genome as described previously[10, 22]. PCR amplicons were purified
79 using the NucleoSpin Gel and PCR Clean-up from Machery Nagel (Germany) before sending for
80 commercial sequencing.

81 # Enterovirus typing and phylogenetic analysis

82 The HEV identity was investigated by searching the partial VP1 sequences obtained in this study
83 in comparison with the HEV prototypes and other HEVs sequences available in GenBank using
84 basic local alignment (BLAST, http://www.ncbi.nlm.nih. gov/BLAST). The HEV that showed
85 more than 75% nucleotide similarity was assigned to be the same genotype. Phylogenetic analyses
86 of the RNA sequences of E18 and CVB5 genotypes were conducted based on the neighbor-joining
87 (NJ) methods with Tamura-Nei and Kimura 2-parameter models implemented in MEGA 6 with
88 1000 bootstraps replicates for branch confidence in clades in each tree (Tamura et al., 2013).
89 Poliovirus was used as an out-group.

90 # Recombination analysis

91 The aligned E18 and CVB5 sequences were tested for any possible recombination events using
92 the RDP 4 software [23]. All seven default statistical tools were employed including PHI statistic
93 (Φ_w. or pairwise homoplasy index, PHI)[24], Geneconv [25], Bootscan [26], Max X^2 [27],
94 Chimaera [28], SiScan, and 3Seq [29]. P-value differs depending on the recombination test used,

number of sequences analyzed where P-value is subsequently corrected by the RDP 4 default Bonferroni-correction. The recombination tests were performed using default settings and a Bonferroni-corrected *P* value cutoff of 0.05. The recombination analyses were confirmed by SplitTree 4 (ver. 4.14.6) software [30] using PHI statistic [24] and DnaSP 5.1 software [31].

Genetic diversity analysis and neutrality tests

To account for the limited number of genotype HEV cases (26) and the intermediate length of VP1 sequence (400bp) and the diversity that might be caused external factors such as amplifications and sequencing, several diversity indices were used [32]. The genetic diversity of the 13 E18 and the 11 CVB5 (9 in the present study and the 2 previously detected in 2013) detected in Palestine were analyzed based on VP1 gene. The 27 E18 from 10 different countries reported in the period of 1999-2017 and 25 CVB5 from 13 different countries reported in the period between 1971 and 2017 were retrieved from the Genebank and included in the analysis. EV18 and CVB5 RNA sequences were separately aligned using MEGA 6 [33]. Then, the genetic diversity of the VP1 gene in both E18 and CVB5 genotypes were calculated based on parameters including haplotype diversity (H_d), nucleotide diversity (π). Haplotype diversity (or gene diversity) refers to the number of haplotypes in the population. Nucleotide diversity is the average number of nucleotide differences per site in pairwise comparison between RNA sequences[34]. To detect departure from neutral theory of evolution (random mutation) at a constant population size, two neutrality tests were used. The first was Tajima's *D* test which compares the differences between the numbers of segregating sites (S) and the average number of nucleotide differences between two randomly chosen sequences from within in the population (K)[35]. The second test was Fu and Li's *F* test which compares differences between the number of singleton mutations and the average number

of nucleotide differences between pairs of sequences [36]. DnaSP 5.1 software was used for the calculation at default settings[37].

Statistical analysis

Epidemiologic data were analyzed using EpiInfo statistical package. Analysis included distribution, 2x2 tables, and frequency tables. Fisher's exact test and Chi square with 95% confidence interval were calculated. The level of statistical significance was $P<0.05$.

Results

Demography of circulating EVs

A total of 249 CSF samples were collected from hospitalized patients suspected of having aseptic meningitis in the period between March-- October, 2017. Overall, 54/249 (22%) yielded positive results for HEV using RT-PCR targeting the 5'UTR. The general characteristic and the clinical history of the study samples are shown in table 1.

Table 1. Demographic data and clinical history of the study samples

	Total 249 No. (%)	**EV 54**	**Non-EV 195**
Sex:			
Male	156 (62.5)	33	123
Female	93 (27.5)	21	72
Age in month, median (range)	6 (0-92)	7 (0-92)	6 (0-90)
Place of residence:			
Nablus	156 (62.6)	26 (48.1)	130 (66.7)
Jenin	93 (37.4)	28 (51.9)	65 (33.3)
Pleocytosis	50/179 (28)	13 (26)	37 (74)
Fever	233/249 (93.6)	179 (77)	54 (23)
Headache	66/249 (26.5)	33 (50)	33(60)
Diarrhea	53/249 (21.3)	20/54 (37)*	33/195
Vomiting and Nausea	82/249 (32.9)	26/54*	56/195
Stiff neck	18/249 (7.2)	13/41*	5/195
Photophobia	9/249 (3.6)	7/54 (13)*	2/195
Mental disturbances	5/249 (2)	2 (40)	3(60)

* $P < 0.05$

Twenty-six (48%) were successfully genotyped by sequencing the amplicon of the amplified 5' VP1 region. Four different types of HEVs were detected. All of them belong to HEV-B species. Thirteen of the detected HEVs (50%) were E18, 9 (34.5%) were coxsackievirus B5 (CVB5), 3 (11.5%) were E25 and 1 (3.8%) was CVB2. Demographic data, the clinical history and the gene Bank accession number of the genotypes HEV cases are shown in Table 2.

155 **Table 2. Demographic data and clinical characteristic of the diagnosed HEVs genotypes**

Sample No.	Accession No.	EV genotype	Month/year of isolation	Age (month)	District
170J/017	MG773498	E18	04/2017	33	Jenin
186J/017	MG773499	E18	05/2017	64	Jenin
188J/017	MG773500	E18	05/2017	16.5	Jenin
208N/017	MG773501	E18	05/2017	70	Nablus
227J/017	MG773502	E18	07/2017	57	Jenin
232J/017	MG773503	E18	08/2017	3	Jenin
247J/017	MG773504	E18	07/2017	5	Jenin
257J/017	MG773505	E18	08/2017	81	Jenin
262J/017	MG773506	E18	08/0217	67	Jenin
265J/017	MG773507	E18	08/2017	3	Jenin
344J/017	MG773508	E18	04/2017	6	Jenin
376J/017	MG773509	E18	09/2017	77	Jenin
380J/017	MG773510	E18	08/2017	10.5	Jenin
206N/017	MG773497	CVB5	05/2017	40	Nablus
230J/017	MG773489	CVB5	07/2017	75.5	Jenin
211N/017	MG773511	CVB2	05/2017	<1	Nablus
260J/017	MG773490	CVB5	06/2017	<1	Jenin
338N/017	MG773491	CVB5	04/2017	4	Nablus
342J/017	MG773492	CVB5	05/2017	1	Jenin
345J/017	MG773495	CVB5	05/2017	21	Jenin
354N/017	MG773496	CVB5	04/2017	35	Nablus
407N/017	MG773494	CVB5	03/2017	1	Nablus
433N/017	MG773493	CVB5	04/2017	49	Nablus
261/017	MG773514	E25	05/2017	42	Jenin
299N/017	MG773512	E25	06/2017	6	Nablus
389J/017	MG773513	E25	09/2017	51	Jenin

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157 # Phylogenetic analysis of the partial VP1 gene of the E18 and CVB5

158 Phylogenetic analysis of the partial VP1 gene was conducted using the thirteen E18 and eleven
159 CVB5 strains from Palestine (9 in 2017 and 2 in 2013). Twenty-seven E18 and 25 CVB5 sequences
160 were retrieved from GenBank were included for comparison. The phylogenetic tree for E18
161 showed three main clusters with all Palestinian isolates uniquely clustering together along with
162 those from China. Similarly, The CVB5 isolates were distributed into three clusters with
163 Palestinian isolates in 2017 clustering together, along with isolates from different areas of the
164 world, whereas, the two Palestinian isolates in 2013grouped within the Predominantly-Chinese
165 cluster (Figure. 1)

Figure 1. Bootstrap consensus (1000 replicates) neighbor joining (NJ) phylogenetic dendrograms constructed based on the partial VP1 gene of E18 (left) and CVB5 (right). Dendrograms for E18 (○) and CVB5 (□) are plotted against the global distribution of the geographical origin of the isolates. The two cluster contain strains from Palestine (Δ) along with strains from GenBank. Poliovirus from vaccine was included as an out-group.

Recombination analysis

The recombination analysis did not find any statistically significant evidence for recombination events between the aligned sequences, both on the Palestinian strains level (13 E18 strains and 11 CVB5) and international level that included Palestinian strains pooled with strains from the Gene bank (40E18 strains and 36CVB5). However, minimum number of recombination events was detected by DnaSP 5.1 software for E18 and CVB5. RDP4 software (PHI statistic) revealed good chance of recombination, but at a very low P-value (0.00001).

Diversity indices

Population nucleotide diversity indices and neutrality tests were calculated for the partial VP1 gene for E18 and CVB5, separately, based on phylogenetic clusters (Tables 3 and 4). The totally haplotype diversity (Hd) for the 40 E18 sequences was 0.98± 0.01 and 0.99± 0.006 for the 36CVB5. At the same time, the total genetic diversity (π) for E18 was 0.12± 0.02 and 0.17± 0.01 for CVB5. The average number of nucleotide differences between any two sequences (k) for E18 and CVB5 were 35.5 and 41, respectively. Cluster I of E18 showed peculiar results compared to the other two clusters. In this group, we detected 12 haplotypes in 19 sequences with the lowest haplotype-to-sequence (h:n) ratio (0.6:1), compared to equal h:n ratio for clusters II and III. Haplotype diversity (H_d), nucleotide diversity (π), and number of segregating (polymorphic) sites (S) for E18 were lowest in cluster I which is comprised mainly of Palestinian strains (13/19) along

with those from China; confirming low level of genetic diversity present in this cluster compared to clusters II and III. Tajima's D and Fu-Li'sF tests were negative in cluster I and showing statistically significant departure from neutrality (random mutation) ($P<0.01$). As for CVB5 genotype, cluster III recorded the lowest genetic diversity indices and neutrality tests with negative but insignificant values (Table 3 and 4). Both E18 and CVB5 showed a combination of high haplotype diversity (Hd), but low genetic diversity (π). Also, both showed overall negative values of neutrality tests; Tajima's D and Fu-Li's F.

Table 3. Haplotype-nucleotide diversity and neutrality tests of three cluster of E18 as calculated for the VP1 gene

	Haplotype- nucleotide diversity							Neutrality tests	
Cluster	n	h	h:n	Hd±SD	π±SD	K	S	Tajima's D	Fu-Li's F
RED-I	19	12	0.6:1	0.92± 0.05	0.03± 0.01	11.3	67	-1.80*	-2.97*
Green-II	7	7	1:1	1.0± 0.08	0.07± 0.01	61.9	165	-0.57	-0.56
Blue-III	10	9	0.9:1	1.0± 0.05	0.06± 0.01	55.2	171	-0.93	-1.12
Total	**41**	**34**	**0.9:1**	**0.98± 0.01**	**0.12± 0.02**	**35.5**	**184**	**-1.50**	**-2.80**

n**:** Number of sequences, h: Number of Haplotypes, Hd: Haplotype (gene) diversity, π: Nucleotide diversity (per site),K: Average number of nucleotide differences between two randomly chosen sequences from within in the population, S: Number of variable/segregating sites.(1 outgroup, 4 have no clear cluster). *: P<0.01.

Table 4. Haplotype/nucleotide diversity and neutrality tests of three cluster of CVB5 as calculated for the VP1 gene

	Haplotype nucleotide diversity							Neutrality tests	
Cluster	n	h	n:h	Hd±SD	π±SD	K	S	Tajima's D	Fu-Li's F
Blue-I	14	14	1:1	1.0± 0.03	0.13± 0.009	89.3	89	0.05	0.36
Green-II	13	13	1:1	1.0± 0.03	0.10± 0.01	28.3	74	-0.257	-0.27
RED-III	19	18	1:1	0.94± 0.02	0.08± 0.007	24.3	75	-0.55	-1.13
Total	**46**	**45**	**1:1**	**0.99± 0.006**	**0.17± 0.01**	**41.04**	**133**	**-0.75**	**-1.76**

n**:** Number of sequences, h: Number of Haplotypes, Hd: Haplotype (gene) diversity, π: Nucleotide diversity (per site), K: Average number of nucleotide differences between two randomly chosen sequences from within in the population, S: Number of variable/segregating sites. (1 outgroup, 4 have no clear cluster)

Inter-population pairwise genetic distance (Fst) in the three E18 populations ranged from 0.39 to 0.63 with Nm value from 0.29 to 0.78 (Table 5 and 6). Fst for cluster I containing all Palestinian strains compared to clusters II and III were high (0.51 and 0.63) (positive Tajima's D) indicating population differentiation with low gene flow, Nm (0.48 and 0.29). However, genetic

differentiation between clusters II and III is low (Fst=0.39) with high Nm (0.78), but still reflected genetic differentiation. This is supported by the negative value of Gst (-0.006).

As for CVB5, cluster III which contained most of the Palestinian strains is differentiated from the other two clusters, I and II, as reflected by the high Fst and low Nm values (Fst=0.54, Nm=0.43) and (Fst=0.49 and 0.52), respectively. At the same time, the genetic differentiation between clusters I and II is relatively low as supported by very low Gst value (Gst=0.00005) and relatively low Fst (0.32) with high gene flow (Nm=1.06).

Table 5: Gene flow and genetic differentiation indices between the three E18 clusters estimated from VP1 sequences

Phylogroup 1	Phylogroup 2	Fst	Nm	Kxy	Dxy	Gst	Da
Red-I	Green-II	0.51	0.48	30.6	0.09	0.029	0.05
Red-I	Blue-III	0.63	0.29	39.7	0.13	0.024	0.08
Blue -III	Green-II	0.39	0.78	30.2	0.09	-0.006	0.04

Fst: Wright's F-statistics, pairwise genetic distance, Nm: Gene flow and population migration among populations which is calculated as Nm=(1-Fst)/2Fst, Kxy: Average proportion of nucleotide differences between populations. Dxy: The average number of nucleotide substitutions per site between populations, Da: The number of net nucleotide substitutions per site between populations, Gst: Genetic differentiation index based on the frequency of haplotypes.

Table 6: Gene flow and genetic differentiation indices between the three CB5 clusters estimated from VP1 sequences

Phylogroup 1	Phylogroup 2	Fst	Nm	Kxy	Dxy	Gst	Da
Red-III	Green-II	0.54	0.43	48.8	0.19	0.005	0.11
Red-III	Blue-I	0.49	0.52	51.8	0.21	0.005	0.10
Blue-I	Green-II	0.32	1.06	43.9	0.18	0.00005	0.06

Fst: Wright's F-statistics, pairwise genetic distance, Nm: Gene flow and population migration among populations which is calculated as Nm=(1-Fst)/2Fst, Kxy: Average proportion of nucleotide differences between populations Dxy: The average number of nucleotide substitutions per site between populations, Da: The number of net nucleotide substitutions per site between populations, Gst: Genetic differentiation index based on the frequency of haplotypes.

Discussion

So far, only one report in Palestine confirmed the isolation of 7 different enteroviruses genotypes including echovirus 13, 14, 9, 30, 16, 6 and coxsackievirus B5 from sporadic cases of aseptic meningitis and/or sepsis like illness[10]. In the present study, all of the HEV positive cases occurred in children less than 7 years old and 50% of them < 1-year-old. Highest rate of enterovirus-positive cases was reported previously in children less than 1 year of age in Palestine, United States, and Korea [10, 38]. On the contrary, other studies, reported that HEV aseptic meningitis occurs most frequently in patients with age range of 3–12 years' old [13-15, 39]. The discrepancy in the HEV age groups could be due to the source of cases whether sporadic or from an outbreak.

The present study showed that E18 and CVB5 were the most predominant genotypes, representing, 50% and 34.6% respectively. Several recent studies in Germany, Taiwan, Korea, and China reported that both E18 and CVB5 were the most predominant HEVs reported from sporadic and outbreak cases of aseptic meningitis [13, 15-16, 40].

All E18 sequences included in the phylogenetic analysis grouped into three major genetic clusters. The E18 isolates from Palestine clustered together along with those from China reported in 2015 in cluster I. Clusters II and III contain E18 from Australia, France, USA, Sweden, Russian, Germany and Netherlands reported in the periods 2005- 2012 and 1999-2012, respectively. Other minor clusters included cluster IV and V which included the prototype Metacalf and few HEV reported from India and China in the period 2000-2008. HEV types in clusters IV and V may have circulated during a limited period and then disappeared. Similar results reported a genetic divergence in the complete VP1 gene of the E18 that resulted in the formation of five phylogenetic clusters [40]. These viruses were reported recently from China, India, South Korea, Australia,

Netherlands, Germany, Sweden, Russia and France [40]. Phylogenetic analysis of partial or complete VP1 gene of E11, E30, E13, and E6 of the HEV-B species revealed high genetic diversity showing several clusters and sub-clusters [40]. Accordingly, such data indicate that the same HEV genotype may circulate in different geographies at different times. Therefore, and due to lack of reporting HEVs in Palestine, the possibility of knowing whether E18 isolates in cluster I are recently introduced or have already been circulating before remains a dilemma.

The CVB5 sequences grouped into three genetic clusters (Fig 1). Cluster III contained the 9 Palestinian strains isolated in 2017 along with CVB5 from Brazil, France, Cyprus, Germany and Australia reported in the period from 2006 to 2017. The 2 Palestinian isolates in 2013 grouped with a more recent CVB5 in cluster II (2003-2014) from China, Denmark, France and Netherland. Cluster I contained the prototype Faulkner and isolates from France, Germany, Japan, Korea, China, Hong Kong, India, Finland and Sweden. Recently, few studies compared the partial or the complete VP1 gene revealing genetic diversity of CVB5 in 2-5 clusters [41-43]. Accordingly, the findings in the present study reaffirm that two or more clusters may co-circulate and coevolve in the same region as a result of the genetic diversity forces such as mutation or recombination.

The recombination for both species E18 and CVB5 was minimal, if at all present between the study sequences which could be explained by the infrequency of recombination within the capsid region, VP1 [19-20]. This puts forward mutation rates as the main cause of genetic diversity. The significant departure from neutrality as confirmed by negative Tajima's D and Fu-Li's F accompanied by high haplotype (Hd) and low nucleotide diversity (π) for cluster I of E18 (Table3) may suggest recent rapid population expansion phase following bottleneck or genetic hitchhiking (genetic draft or gene sweep) that bring excess number of rare alleles. This was supported by the negative values Tajima's D and Fu-Li's F tests in the individual populations and the overall value

for the three populations. A study in Taiwan showed that partial VP1 genes of E18 have low genetic diversity with high similarity between regions [14]. The clustering of the Palestinian and Chinese isolates in cluster I could have been brought about by the activity of Palestinian traders to China and back. Cluster II and III had similar diversity indices, but without any significance.

Cluster III of CVB5 showed lower genetic diversity than the others (Table 4), yet insignificant; suggesting that cluster III may have undergone a neutral or contraction period or may be due to small subpopulations with limited number of sequences to yield sufficient statistical power. A study from Korea revealed very high similarity in the partial VP1 sequences among Korean samples (intra-population similarity) and between Chinese strains (inter-population similarity) [16]. The genetic similarity of the Palestinian, Korean and Taiwanese E18 and CVB5 strains to those from China, may hint to their dispersal from China to the rest of the world [14, 16]. However, a more extensive study of several endemic areas should be conducted to confirm the ancestral origin of E18 and CVB5, the route of spread and the distribution.

The estimations of inter-population comparison indices (Fst, Nm, Kxy, Dxy, Gst and Da) (Tables5 and 6) support high level of genetic differentiation between the three main clusters of both E18 and CVB5. These results are supportive of the cluster in phylogentic analyses (Fig1).

In conclusion, the present study unravels three main clusters for each of the E18 and CVB5 with both showing high haplotype diversity compared to lower genetic diversity. The Palestinian isolates grouped mainly into one cluster for each viral species. Finally, present study supports close genetic relationship between Palestinian HEV species as confirmed by population genetics and phylogenetic analyses.

REFERENCES

[1] Knowles NJ, Hovi T, Hyypiä T, King AMQ, Lindberg AM, Pallansch MA. Virus taxonomy: classification and nomenclature of viruses: ninth report of the international committee on taxonomy of viruses. In: King AMQ AM, Carstens EB, Lefkowitz EJ,, editor. Picornaviridae. San Diego: Elsevier; 2012. p. 885-80.

[2] Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. Typing of human enteroviruses by partial sequencing of VP1. J Clin Microbiol. 1999;37:1288-93.

[3] Harvala H, Calvert J, Van Nguyen D, Clasper L, Gadsby N, Molyneaux P, et al. Comparison of diagnostic clinical samples and environmental sampling for enterovirus and parechovirus surveillance in Scotland, 2010 to 2012. Euro Surveill. 2014;19.

[4] Othman I, Volle R, Elargoubi A, Guediche MN, Chakroun M, Sfar MT, et al. Enterovirus meningitis in Tunisia (Monastir, Mahdia, 2011-2013): identification of virus variants cocirculating in France. Diagn Microbiol Infect Dis. 2016;84:116-22.

[5] Xiao H, Guan D, Chen R, Chen P, Monagin C, Li W, et al. Molecular characterization of echovirus 30-associated outbreak of aseptic meningitis in Guangdong in 2012. Virol J. 2013;10:263.

[6] Logotheti M, Pogka V, Horefti E, Papadakos K, Giannaki M, Pangalis A, et al. Laboratory investigation and phylogenetic analysis of enteroviruses involved in an aseptic meningitis outbreak in Greece during the summer of 2007. J Clin Virol. 2009;46:270-4.

[7] Glaser CA, Honarmand S, Anderson LJ, Schnurr DP, Forghani B, Cossen CK, et al. Beyond viruses: clinical profiles and etiologies associated with encephalitis. Clin Infect Dis. 2006;43:1565-77.

[8] Laxmivandana R, Yergolkar P, Gopalkrishna V, Chitambar SD. Characterization of the non-polio enterovirus infections associated with acute flaccid paralysis in South-Western India. PLoS One. 2013;8:e61650.

[9] Lang M, Mirand A, Savy N, Henquell C, Maridet S, Perignon R, et al. Acute flaccid paralysis following enterovirus D68 associated pneumonia, France, 2014. Euro Surveill. 2014;19.

[10] Dumaidi K, Al-Jawabreh A. Molecular detection and genotyping of enteroviruses from CSF samples of patients with suspected sepsis-like illness and/or aseptic meningitis from 2012 to 2015 in West Bank, Palestine. PLoS One. 2017;12:e0172357.

[11] Chen X, Li J, Guo J, Xu W, Sun S, Xie Z. An outbreak of echovirus 18 encephalitis/meningitis in children in Hebei Province, China, 2015. Emerg Microbes Infect. 2017;6:e54.

[12] Zhu Y, Zhou X, Liu J, Xia L, Pan Y, Chen J, et al. Molecular identification of human enteroviruses associated with aseptic meningitis in Yunnan province, Southwest China. Springerplus. 2016;5:1515.

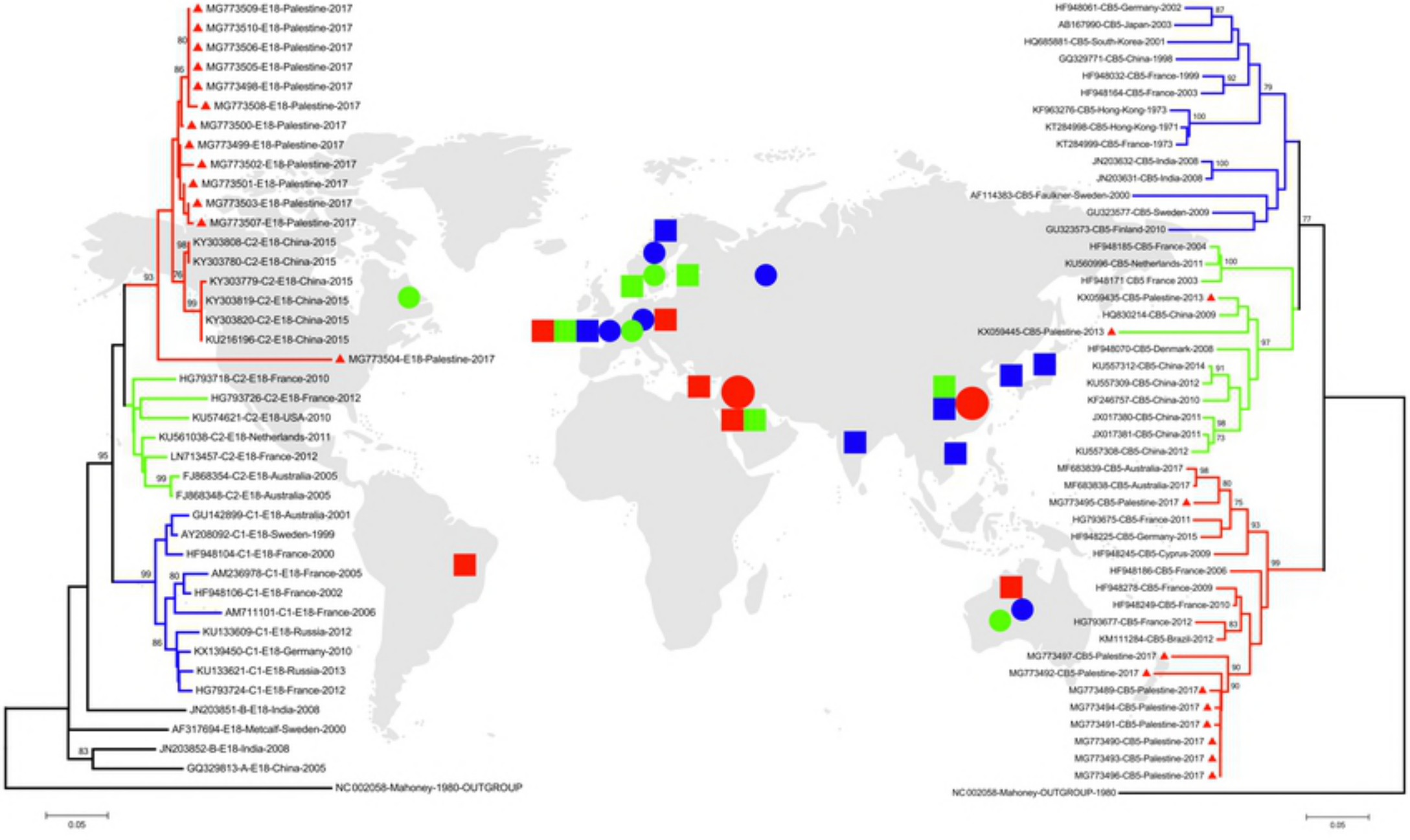
[13] Liu N, Jia L, Yin J, Wu Z, Wang Z, Li P, et al. An outbreak of aseptic meningitis caused by a distinct lineage of coxsackievirus B5 in China. Int J Infect Dis. 2014;23:101-4.

[14] Tsai HP, Huang SW, Wu FL, Kuo PH, Wang SM, Liu CC, et al. An echovirus 18-associated outbreak of aseptic meningitis in Taiwan: epidemiology and diagnostic and genetic aspects. J Med Microbiol. 2011;60:1360-5.

[15] Krumbholz A, Egerer R, Braun H, Schmidtke M, Rimek D, Kroh C, et al. Analysis of an echovirus 18 outbreak in Thuringia, Germany: insights into the molecular epidemiology and evolution of several enterovirus species B members. Med Microbiol Immunol. 2016;205:471-83.

[16] Baek K, Park K, Jung E, Chung E, Park J, Choi H, et al. Molecular and epidemiological characterization of enteroviruses isolated in Chungnam, Korea from 2005 to 2006. J Microbiol Biotechnol. 2009;19:1055-64.
[17] Huang B, Harrower B, Burtonclay P, Constantino T, Warrilow D. Genome Sequences of Coxsackievirus B5 Isolates from Two Children with Meningitis in Australia. Genome Announc. 2017;5.
[18] Muslin C, Joffret ML, Pelletier I, Blondel B, Delpeyroux F. Evolution and Emergence of Enteroviruses through Intra- and Inter-species Recombination: Plasticity and Phenotypic Impact of Modular Genetic Exchanges in the 5' Untranslated Region. PLoS Pathog. 2015;11:e1005266.
[19] Lukashev AN. Role of recombination in evolution of enteroviruses. Rev Med Virol. 2005;15:157-67.
[20] Simmonds P, Welch J. Frequency and dynamics of recombination within different species of human enteroviruses. J Virol. 2006;80:483-93.
[21] Guney C, Ozkaya E, Yapar M, Gumus I, Kubar A, Doganci L. Laboratory diagnosis of enteroviral infections of the central nervous system by using a nested RT-polymerase chain reaction (PCR) assay. Diagn Microbiol Infect Dis. 2003;47:557-62.
[22] Thoelen I, Lemey P, Van Der Donck I, Beuselinck K, Lindberg AM, Van Ranst M. Molecular typing and epidemiology of enteroviruses identified from an outbreak of aseptic meningitis in Belgium during the summer of 2000. J Med Virol. 2003;70:420-9.
[23] Martin D, Rybicki E. RDP: detection of recombination amongst aligned sequences. Bioinformatics. 2000;16:562-3.
[24] Bruen TC, Philippe H, Bryant D. A simple and robust statistical test for detecting the presence of recombination. Genetics. 2006;172:2665-81.
[25] Sawyer, SA. Geneconv: a computer package for the statistical detection of gene conversion. Department of Mathematics. Washington University in St Louis. http://www.math.wustl.edu/sawyer; 1999.
[26] Salminen MO, Carr JK, Burke DS, McCutchan FE. Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning. AIDS Res Hum Retroviruses. 1995;11:1423-5.
[27] Smith JM. Analyzing the mosaic structure of genes. J Mol Evol. 1992;34:126-9.
[28] Posada D, Crandall KA. Evaluation of methods for detecting recombination from DNA sequences: computer simulations. Proc Natl Acad Sci U S A. 2001;98:13757-62.
[29] Martin DP, Williamson C, Posada D. RDP2: recombination detection and analysis from sequence alignments. Bioinformatics. 2005;21:260-2.
[30] Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol. 2006;23:254-67.
[31] Hudson RR, Kaplan NL. Statistical properties of the number of recombination events in the history of a sample of DNA sequences. Genetics. 1985;111:147-64.
[32] Thai KT, Henn MR, Zody MC, Tricou V, Nguyet NM, Charlebois P, et al. High-resolution analysis of intrahost genetic diversity in dengue virus serotype 1 infection identifies mixed infections. J Virol. 2012;86:835-43.
[33] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30:2725-9.

[34] Nei M. Molecular evolutionary genetics. New York: Colombia University Press. 512 p.M. 1987.
[35] Tajima F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics. 1989;123:585-95.
[36] Fu YX, Li WH. Statistical tests of neutrality of mutations. Genetics. 1993;133:693-709.
[37] Rozas J, Ferrer-Mata A, Sanchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, et al. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. Mol Biol Evol. 2017;34:3299-302.
[38] Sawyer MH, Holland D, Aintablian N, Connor JD, Keyser EF, Waecker NJ, Jr. Diagnosis of enteroviral central nervous system infection by polymerase chain reaction during a large community outbreak. Pediatr Infect Dis J. 1994;13:177-82.
[39] Muehlenbachs A, Bhatnagar J, Zaki SR. Tissue tropism, pathology and pathogenesis of enterovirus infection. J Pathol. 2015;235:217-28.
[40] Zhang H, Zhao Y, Liu H, Sun H, Huang X, Yang Z, et al. Molecular characterization of two novel echovirus 18 recombinants associated with hand-foot-mouth disease. Sci Rep. 2017;7:8448.
[41] Gullberg M, Tolf C, Jonsson N, Mulders MN, Savolainen-Kopra C, Hovi T, et al. Characterization of a putative ancestor of coxsackievirus B5. J Virol. 2010;84:9695-708.
[42] Papa A, Skoura L, Dumaidi K, Spiliopoulou A, Antoniadis A, Frantzidou F. Molecular epidemiology of Echovirus 6 in Greece. Eur J Clin Microbiol Infect Dis. 2009;28:683-7.
[43] Rezig D, Fares W, Seghier M, Yahia AB, Touzi H, Triki H. Update on molecular characterization of coxsackievirus B5 strains. J Med Virol. 2011;83:1247-54.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