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Molecular characterization of enterovirus 71 sibling strains for thermal adaption in Vero cells with adaptive laboratory evolution

Heng Chen[a,b], Yue Cheng[b], Xian Liang[b], Jian-Tong Meng[b], Hao-Jiang Zuo[a], Li-Yuan Su[b], Xi-Xi Wang[b], Cai-Bin Yang[b], Rong-Sheng Luan[a,*]

[a] West China School of Public Health, & No. 4 West China Teaching Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
[b] Chengdu Center for Disease Control and Prevention, Chengdu 610041, Sichuan Province, China

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ABSTRACT

Enterovirus 71 is the main pathogen that causes severe and fatal hand-foot-mouth-disease (HFMD) cases. As the enterovirus virus mutation has implications for pathogenesis, vaccine development, antiviral therapy, and epidemiological disease management of the virus. In this study, we investigated the variations of enterovirus 71 in thermal adaption, using the method of adaptive laboratory evolution. The sibling virus strains were isolated from a 2-year-old severe case of HFMD (#100) and her symptomless close contact (#101). Both strains were cultured in Vero cells by serial passage of 36 generations at the temperatures of 28.0 °C, 33.0 °C and 39.5 °C to construct adaptive lineages. According to the comparative analysis of phenotypes between adapted strains and parental strains, differences in growth rate were observed in the sibling lineages and a larger plaque was found mainly in the hot adapted strains for lineage #101. Two sets of adaptive strains from six time points (parental, 12th 17th, 31st, 35th passage and endpoint) were sequenced and analyzed by both Sanger sequencing and Next Generation Sequencing. Several variations in most coding genes and one reverse mutation in 5′UTR was observed, along with the identity of 99.8% for complete genome for both lineages. Notably, thermal specific non-synonymous mutations were found in the gene of VP1\VP3\3A\2C\3C. Moreover, the concurrent mutations A292G, A434G and A355C/T of sibling lineages in VP1 showed quantificational trace with distinguishing patterns for different temperatures, which were suspected to be the thermo-sensitive mutation hotspots. These results highlight the possible rules of thermal adaption in enterovirus 71, produce a novel picture of genome evolution of the virus, and shed light on viral variation and evolution.

1. Introduction

Enterovirus 71 (EV71) is a positive single-stranded RNA virus that belongs to the *Picornaviridae* family, genus *Enterovirus*, species Enterovirus A. It is responsible for severe cases even death of Hand-Foot-Mouth-Disease (HFMD), thus considered the most important neurotropic enterovirus after poliomyelitis (Lin et al., 2015). In the past thirty years, enterovirus 71 has caused several large epidemics worldwide and threatened the Public Health especially in Asia-Pacific region (Ho et al., 1999; McMinn, 2002; Zhang et al., 2010).

As the genetic variation and recombination were reported to be responsible for the outbreaks (Shih et al., 1998; Zhang et al., 2010), the variants of the EV71 became a great challenge to decipher. This also had wide implications for the virulence, antigenicity and drug resistance of the virus (Combe and Sanjuan, 2014). The vaccine for EV71 has been applied in China with considerable protection for children (Yi et al., 2017; Zhu et al., 2013). However, the subgenotype C4 mainly circulates in mainland China has a mutation rate as frequent as 4.36×10^{-3}/bp annually (Zhang et al., 2013), which makes the threat of EV71 should not be ignored due to the influence of viral variations.

Thermal adaption is important for natural selection and evolution. Cold adaption is well known for attenuated strains of poliovirus, influenza and rubella in the application of vaccines (Jin et al., 2003; Sabin et al., 1954).Two similar viruses (related bacteriophages φX174 and G4) were reported for hot adaption at a high temperature of 44 °C with the nucleotide changes (Holder and Bull, 2001). For HFMD, the high temperature (or fever) increased the incidence of disease (Urashima et al., 2003; Yin et al., 2016) and the risk of severe cases (Zhang et al., 2017). As the temperature sensitivity is related to the virulence of EV71 (Arita et al., 2005; Kung et al., 2007), we hypothesize that thermal adaption is likely to be an important way for the evolution and variation of EV71.

* Corresponding author.
E-mail address: luan_rs@scu.edu.cn (R.-S. Luan).

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In the past, it was difficult to observe the evolution and variation of virus in nature. Fortunately, the development of experimental evolution made it possible for us to understand and even predict the virus variations accurately (Ogbunugafor et al., 2009). Adaptive laboratory evolution (ALE) is an experimental method which aims to gain insight into the basic mechanisms of molecular evolution and adaptive changes in microbial populations during long term selection under specified growth conditions (Dragosits and Mattanovich, 2013). Many important insights into nutrient and stress metabolism of relevant model species have been acquired by ALE (Barrick et al., 2009; Ibarra et al., 2002). Besides, Next Generation Sequencing (NGS) became less costly, making it easier to identify and quantify mutations, to create a clear picture of the variation and evolution of viruses and other organisms (Lang and Desai, 2014).

In this study, ALE was utilized to investigate the molecular characterization of enterovirus 71 sibling strains for thermal adaption by serial passage in Vero cells. The mutations during the adaptive passage at different temperatures from certain time points were measured to evaluate the variation and evolution of enterovirus 71.

2. Materials and methods

2.1. Virus strains

The original EV71 strains were isolated from a severe case of HFMD (female, 2-year-old, labeled as #100) and her asymptomatic close contact (the mother of the patient, 23-year-old, labeled as #101) in Chengdu in 2011. The swab samples were collected and detected by fluorescent PCR and the isolates were confirmed by gene sequencing of VP1. The NGS results confirmed the high homology (99.3% in genome and 100% in VP1) between the virus strains #100 and#101. The strains were cultured and proliferated as sibling strains in Vero cells (green monkey kidney cells, Haling Biotechnology Co., Ltd., Shanghai, Lot: HL-3030) for the following use.

2.2. Cell culture and thermal adaption

Vero cells were routinely maintained in 1640 medium with L-Glutamine and 5% fetal bovine serum (FBS, Gibco, Austria), then incubated at 37.0 °C in 5% CO_2. The primary sibling strains were cultured in Vero cells by serial passage in 6-well plates. A monolayer of Vero cells (~80%–90% confluence) was seeded with 260ul of virus (approximately 10^5 pfu/0.1 ml), cultured in 1640 medium containing L-Glutamine and 2% fetal bovine serum and incubated at 33.0 °C in 5% CO_2. The virus was harvested by freeze-thaw cycle when more than 90% cells showed the typical enteroviral cytopathic effect (CPE), and was then kept at −80 °C for further experiments.

After 12-round serial passage at moderate temperature (33.0 °C), the sibling strains were cultured at different temperatures respectively for thermal adaption till passage 36, which means cold adaptive strains at low temperature (cooling gradually from 33.0 °C to 28.0 °C), cell adaptive strains at moderate temperature (33.0 °C) and hot adaptive strains at high temperature (warming gradually from 37.0 °C to 39.5 °C).

2.3. Phenotype of plaque morphology

The parental strains and endpoint strains of thermal adaption (P36, including cold-adapted, cell-adapted and hot-adapted ones) for both lineage #100 and lineage #101 were investigated by plaque assay which has been modified by Renato Dulbecco (Dulbecco and Vogt, 1954).

2.4. Viral growth and thermal adaptability

The parental strains and endpoint strains of thermal adaption were also observed for the growth and thermal adaptability. Viruses with the volume of 260ul (approximately equal titers of 10^5 pfu/0.1 ml) were seeded on Vero cells and incubated at low temperature (28–29 °C), moderate temperature (33–35 °C) and high temperature(39–40 °C) separately, and were then observed with CPE at intervals. More than 95% cells with the typical enteroviral CPE was taken as an endpoint, and the time consumption was recorded in hours for analysis. This was repeated twice more for each experiment.

2.5. PCR amplification

RNA was extracted and amplified for complete genome of EV71 with reasonable overlaps using commercial kits (Promega, American and Tsingke, China), and the 8 pairs of primers were from previous report (Shili et al., 2004) with minor modifications (Table S.1). The PCR products for 28 virus strains of six time points (parental and 12th 17th, 31st, 35th and 36th passage) were collected and identified by electrophoresis of 1.2%, 100v, 30 min.

2.6. Gene sequencing and analysis

Gene sequencing of full-length of enterovirus 71 genome was carried out with PCR products with both Sanger sequencing and NGS, by Chengdu Tsingke Biological Technology Co., Ltd. with 3730XL and Beijing Novogene Bioinformatics Technology Co., Ltd. with illumina Hiseq, respectively.

Taking the strain of SHZH03 (genbank id: AY 465356.1) as reference sequence of enterovirus 71, raw data was assembled and analyzed for the variations of virus, using Contig Express (Vector NTI Suite 6.0) for Sanger sequencing, and CLC (workbench 11.0) with mapping strategy for NGS.

2.7. Quantitative analysis of single nucleotide polymorphisms

Furthermore, the quantitative information of single nucleotide polymorphisms (SNP) was also collected and analyzed. For Sanger sequencing, the quality value of signal peak was used to calculate the proportional ratio of the corresponding nucleotide for the most suspicious mutation sites, while the software CLC provides quantitative information for every nucleotide including SNP sites.

3. Results

3.1. Thermal adaptive strains

In total, two lineages of both the sibling strains (#100 and #101) were obtained with 12 cell-adapted passages from 1st to 12th passages and 24 thermal adaptive passages from 13th to 36th passages.

3.2. Plaque assay

Diversities of plaque morphology were not obvious in lineage #100 and larger plaques were found in the hot adapted strains of lineage #101. (Fig. 1). Repeated results see Fig.S.1.

3.3. Viral growth and thermal adaptability

The time consumptions indicated the differences in viral growth and thermal adaptability among strains. Little difference was found at moderate temperatures, moderate difference at high temperatures and obvious difference at low temperatures (Table 1 and Fig. 2). It was notable the hot-adapted strains grew the slowest at low temperature, so did the cold-adapted strains at high temperature.

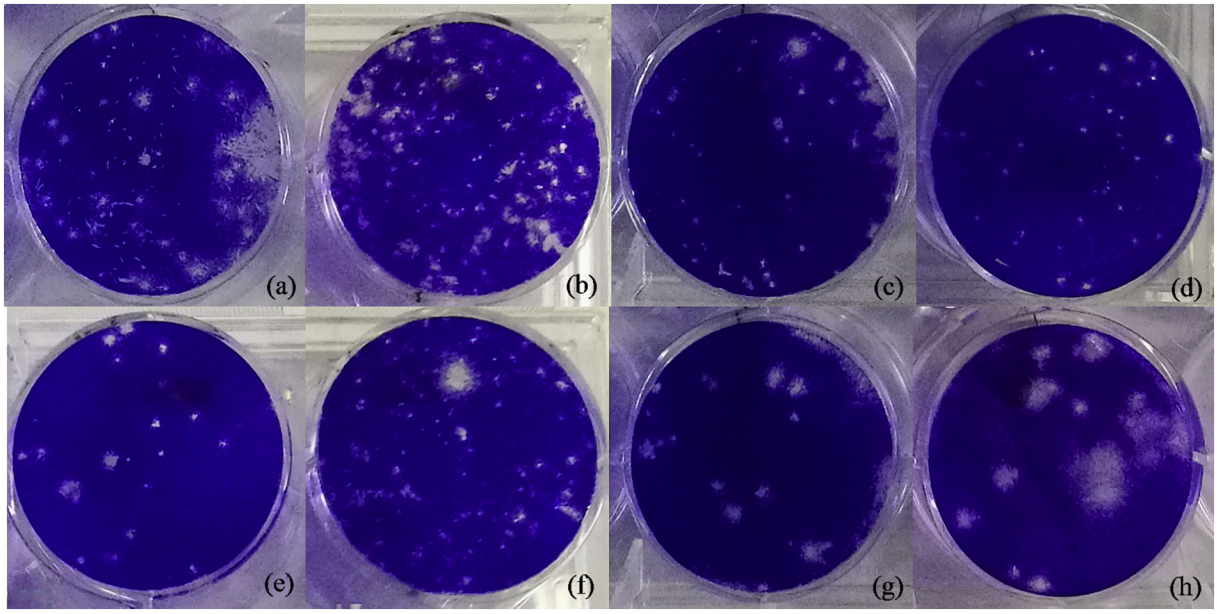

Fig. 1. Plaque morphology of parental strains and thermal-adapted strains of endpoint. lineage #100 (a) parental strain, (b) cold-adapted-P36 strain, (c) cell-adapted-P36 strain and (d) hot-adapted-P36 strain; lineage #101 (e) parental strain, (f) cold-adapted-P36 strain, (g) cell-adapted-P36 strain and (h) hot-adapted-P36 strain.

3.4. Sequence analysis

In total, the amplicons of PCR amplification for 28 virus strains were obtained (Fig.S.2.) and sequenced successfully. Sequences were assembled and submitted to GenBank (accession numbers: MH51181 to MH511208 for Sanger sequencing assembled sequence and 2124768 for NGS raw data). Many variations were found in most cording regions with variable identities. In total, two lineages were with the same identity of 99.8% for the whole genome. The mutations with temperature specificity were often unique in the hot-adapted ones (Tables 2, 3 and 4). Transitions (83.3%, 10/12) occurred much often than transversions (16.7%, 2/12), where the most common transitions are A-G or G-A (58.3%, 7/12).

3.5. SNP and quantification

Among the variations with temperature specificity, the ones of VP1 were consistent between the sibling lineages. The most suspicious mutation sites of G292A, A355C/T, A434G and A844G were with the same nucleotide A. Taking the proportional ratio of nucleotide A as the indicator, it revealed certain regular pattern with gradient trend during serial passage. However, the detectable sites with significant trace were different between Sanger sequencing and NGS, where A292G and A434G were obvious by the former while A355C/T (A355T) and A434G notable by the latter. (Fig. 3.).

4. Discussion

Thermal adaption is vital for orgasms in natural selection and evolution (Dong et al., 2018; Ge et al., 2018). Temperature is often chosen as the stressor in the experimental evolution, and thermal adaptation in viruses. A number of qualitative features of thermal response and adaptation have been found in most studied species and strains of bacteria (Chen and Shakhnovich, 2010; Cullum et al., 2001). Because of the close relationship between temperature and EV71 virus, the thermal adaption of EV71 was proposed and investigated in this study.

As for the phenotypes, variations were found in growth rate for both lineages and plaque morphology in lineage #101. The cold-adapted variations grew the fastest at low temperatures while the slowest at high temperatures. The hot-adapted variations grew the fastest at high temperatures and the slowest at low temperatures. This suggests that the adapted strains gained advantages of the thermal fitness through evolution. The plaque morphology is thought to be related to the temperature sensitivity (Kung et al., 2010), and accompany variations in virulence and antigenicity (Lin et al., 2002). The larger plaque found

Table 1
Time consumption to end-point for virus strains at different culture temperatures.

Strain	28–29 °C(h)				33–35 °C(h)				39–40 °C(h)			
	1st	2nd	3rd	X ± S	1st	2nd	3rd	X ± S	1st	2nd	3rd	X ± S
#100												
Parental	65	108	88	87 ± 22	46	44	48	46 ± 2	46	60	44	50 ± 9
Cold-adapted-P36	41	60	56	52 ± 10	46	44	40	43 ± 3	60	84	60	68 ± 14
Cell-adapted -P36	48	60	60	56 ± 7	46	44	40	43 ± 3	46	48	44	46 ± 2
Hot-adapted-P36	84	108	92	95 ± 12	46	44	44	45 ± 1	46	44	36	42 ± 5
#101												
Parental	85	108	92	95 ± 12	46	44	44	45 ± 1	46	48	44	46 ± 2
Cold-adapted-P36	60	48	56	55 ± 6	46	44	40	43 ± 3	60	84	60	68 ± 14
Cell-adapted -P36	60	60	60	60 ± 0	46	44	40	43 ± 3	46	60	44	50 ± 9
Hot-adapted-P36	100	108	92	100 ± 8	46	44	40	43 ± 3	46	44	36	42 ± 5

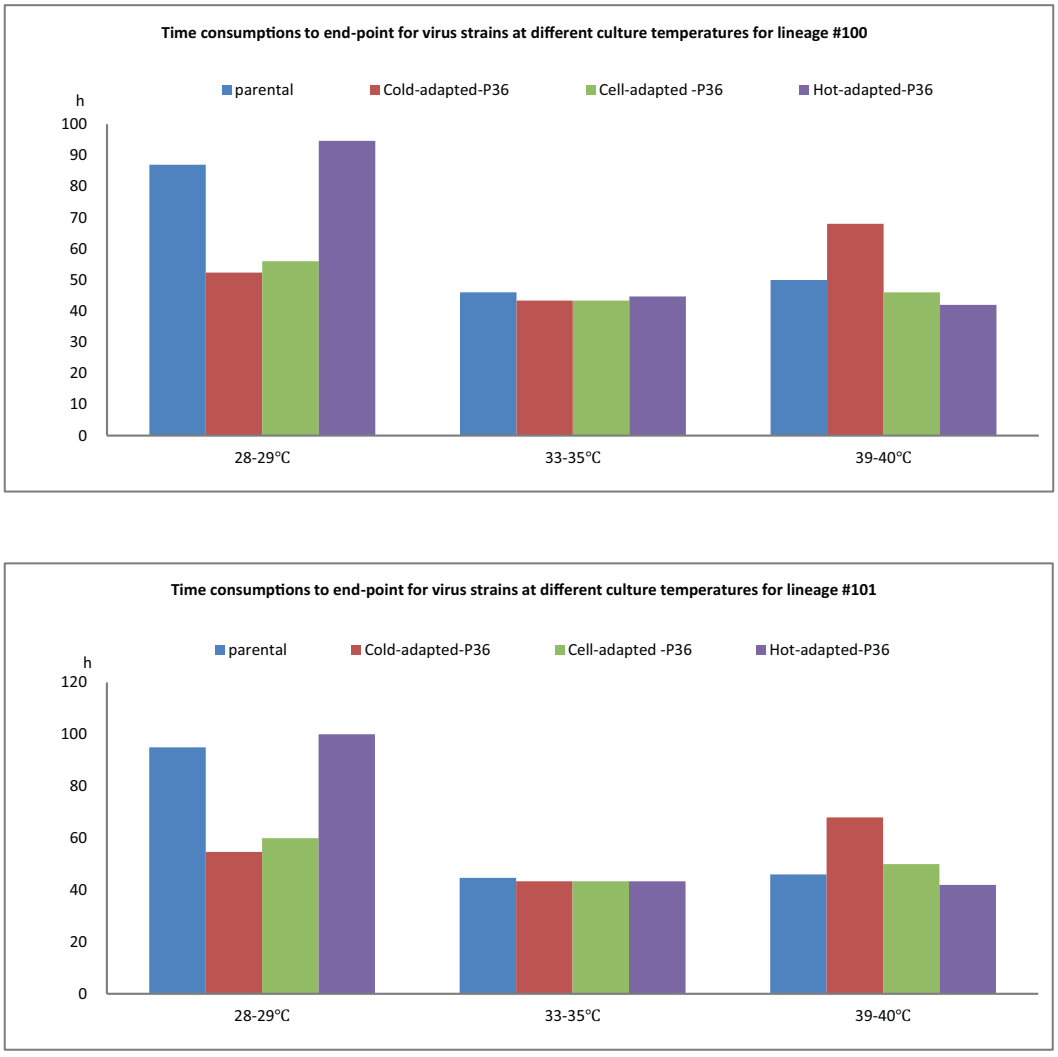

Fig. 2. Time consumptions (in hours) to end-point for virus strains at different culture temperatures for lineage #100 and #101. Parental(blue), clod-adapted-P36(red), cell-adapted-P36(green) and hot-adapted-P36(purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mainly in the hot adapted strains of lineage #101 implied the further influence on the virus from mild case, which needs more experiments to identify in the future.

The variations in genotype were distinguishable between cold adaption and hot adaption. The cell adaption was mostly similar to cold adaption. It was reported that the temperature-resistant strains(Tr) isolated from the severe patients could grow at both 37 °C and 40 °C, while the temperature-sensitive strain (Ts) isolated from mild patient could only grow at 37 °C (Kung et al., 2007). Temperature-sensitive mutants of enterovirus 71 showed attenuation in cynomolgus monkeys (Arita et al., 2005). It suggests that the virulent strains of EV71 characterized with temperature resistance gained evolutional fitness at the high temperature, and the hot adaption may play an important role in the virulence or pathogenicity (Cullum et al., 2001; Holder and Bull,

Table 2
Identity (%) of coding nucleotide and amino acid among parental strains and thermal-adapted strains.

Lineage	Molecular level	VP4	VP2	VP3	VP1	2A	2B	2C	3A	3B	3C	3D
#100												
P12	Nucleotide	100	99.7	100	99.9	100	100	100	100	100	100	100
	Amino acid	100	100	100	99.7	100	100	100	100	100	100	100
P36	Nucleotide	100	98.8	99.7	99.4[a]	99.8	100	99.9	99.2	100	99.8	100
	Amino acid	100	100	99.6	98.3[a]	99.3	100	99.1	97.6	100	100	100
#101												
P12	Nucleotide	100	100	100	100	100	100	100	100	100	100	100
	Amino acid	100	100	100	100	100	100	100	100	100	100	100
P36	Nucleotide	100	100	99.7[a]	99.4[a]	99.8	100	99.9	98.8[a]	100	99.8	100
	Amino acid	100	100	99.2[a]	98.3[a]	100	100	99.7	97.7[a]	100	100	100

[a] With temperature specificity.

Table 3
Predicted amino acid variations in the sequence of thermal-adapted strains (Sanger Sequencing).

Lineage	Gene	Mutation	Virus							
			Parental		Cold-adapted-P36		Cell-adapted-P36		Hot-adapted-P36	
			Amino acid	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid	Codon
#100	VP1	G292A[a]	E	GAG	E	GAG	E	GAG	K	AAG
		A355C/T[a]	M	ATG	L	CTG	L	TTG	M	ATG
		A434G[a]	E	GAA	G	GGA	G	GGA	E	GAA
		A844G	N	AAC	N	AAC	N	AAC	D	GAC
#101	VP3	C85T[a]	H	CAC	Y	TAC	H	CAC	H	CAC
	VP1	A292G[a]	K	AAG	E	GAG	E	GAG	K	AAG
		A355T[a]	M	ATG	L	TTG	M	ATG	M	ATG
		A434G[a]	E	GAA	G	GGA	G	GGA	E	GAA
		A844G[a]	N	AAC	N	AAC	N	AAC	D	GAC
	3A	G202A[a]	V	GTA	M	ATG	V	GTA	V	GTA
		A204G[a]								
		T224C	V	GTG	V	GTG	V	GTG	A	GCG

[a] Also found in the results of NGS.

2001).

Both lineages were genetic stable during the first 12 passage at the moderate temperature. Considerable variations through thermal adaption were found during thermal adapted stage, especially those non-synonymous, temperature-specific in the coding region. It is believed that all essential genes have to satisfy the minimal stability requirement for an organism's survival (Zeldovich et al., 2007).Thus some genes (VP4/2B/3D) were found conserved reasonably during the thermal adaption of EV71. Some variations were distinguishable between different temperatures identified as temperature-specific, which may be induced by the stressor of temperature.

Moreover, mutations that appear in multiple evolution experiments are likely to be drivers of adaptation, but the independently evolved clones share few identical mutations (Lang and Desai, 2014). Therefore, it is remarkable for the variations of A292G and A434G with temperature specificity in both sibling lineages. Moreover, they were also found in other three lineages with original isolates from HFMD cases of different clinic outcomes with its own pattern of gradient changes for SNP (a symptomless, a mild and a death case) (Fig.S.3). It revealed that they were hotspots during the thermal adaption of EV71.Moreover, they were reported as potential virulence determinants in VP1 of enterovirus 71 (Liu et al., 2014).The region spanning amino acids 145–159 corresponding to synthetic peptides have the abilities to induce proliferation of CD4 T cells (Foo et al., 2008), which was related to the antigenicity of enterovirus 71. Possibly, the variations of VP1 induced by temperature would lead to wide influence on the virulence and antigenicity of EV71.

Sanger sequencing with 99.99% accuracy is the “gold standard” for clinical research sequencing. The newer NGS technology is also becoming common due to their higher throughput capabilities, which makes it a more efficient method to identify and quantify mutations.

In this study, both the technologies were applied, for the sequencing strategy of time course sequencing combined with endpoint sequencing, in order to produce complete picture of evolution with all the possibility (increased capacity to generate greater possibilities). By this, it would show when the virus begins to mutate and how to mutate, which makes it possible to predict the variation of virus and develop an efficacious vaccine in timely manner.

Indeed, over a very long time, chance fixation events can affect subsequent evolutionary changes by allowing the future evolution of more complex phenotypes. For instance, the phenotype of Cit + phenotype emerged in the Lenski long-term evolution experiment only after 30,000 generations (Blount et al., 2008). It is important to evaluate the stability and virulence of the adapted strains for a much longer time, as 36 generations are far from enough for evolution. Further the correlation between the fitness and molecular variations is still unclear. Moreover, the results from this study highlight the mutation hot spots and possible rules of thermal adaption in enterovirus 71 and produce a primary picture of genome evolution of the virus, while shedding light on the study of viral variation and evolution.

Table 4
Predicted amino acid variations in the sequence of thermal-adapted strains (by NGS).

Lineage	Gene	Mutation	Virus							
			Parental		Cold-adapted-P36		Cell-adapted-P36		Hot-adapted-P36	
			Amino acid	Codon	Amino acid	Codon	Amino acid	Codon	Amino acid	Codon
#100	VP1	G292A	E	GAG	E	GAG	E	GAG	K	AAG
		A355C/T	M	ATG	L	CTG	L	TTG	M	ATG
		A434G	E	GAA	G	GGA	G	GGA	E	GAA
	2C	G121A	G	GGG	R	AGG	R	AGG	G	GGG
	3A	T196G	S	TCC	A	GCC	A	GCC	S	TCC
#101	VP3	C85T	H	CAC	Y	TAC	H	CAC	H	CAC
	VP1	A292G	K	AAG	E	GAG	E	GAG	K	AAG
		A355T	M	ATG	L	TTG	M	ATG	M	ATG
		A434G	E	GAA	G	GGA	G	GGA	E	GAA
		A844G	N	AAC	N	AAC	N	AAC	D	GAC
	2C	A191G	E	GAA	G	GGA	E	GAA	E	GAA
	3A	G202A	V	GTA	M	ATG	V	GTA	V	GTA
		A204G								
	3C	A528G*	R	AGA	R	AGA	R	AGA	R	AGG

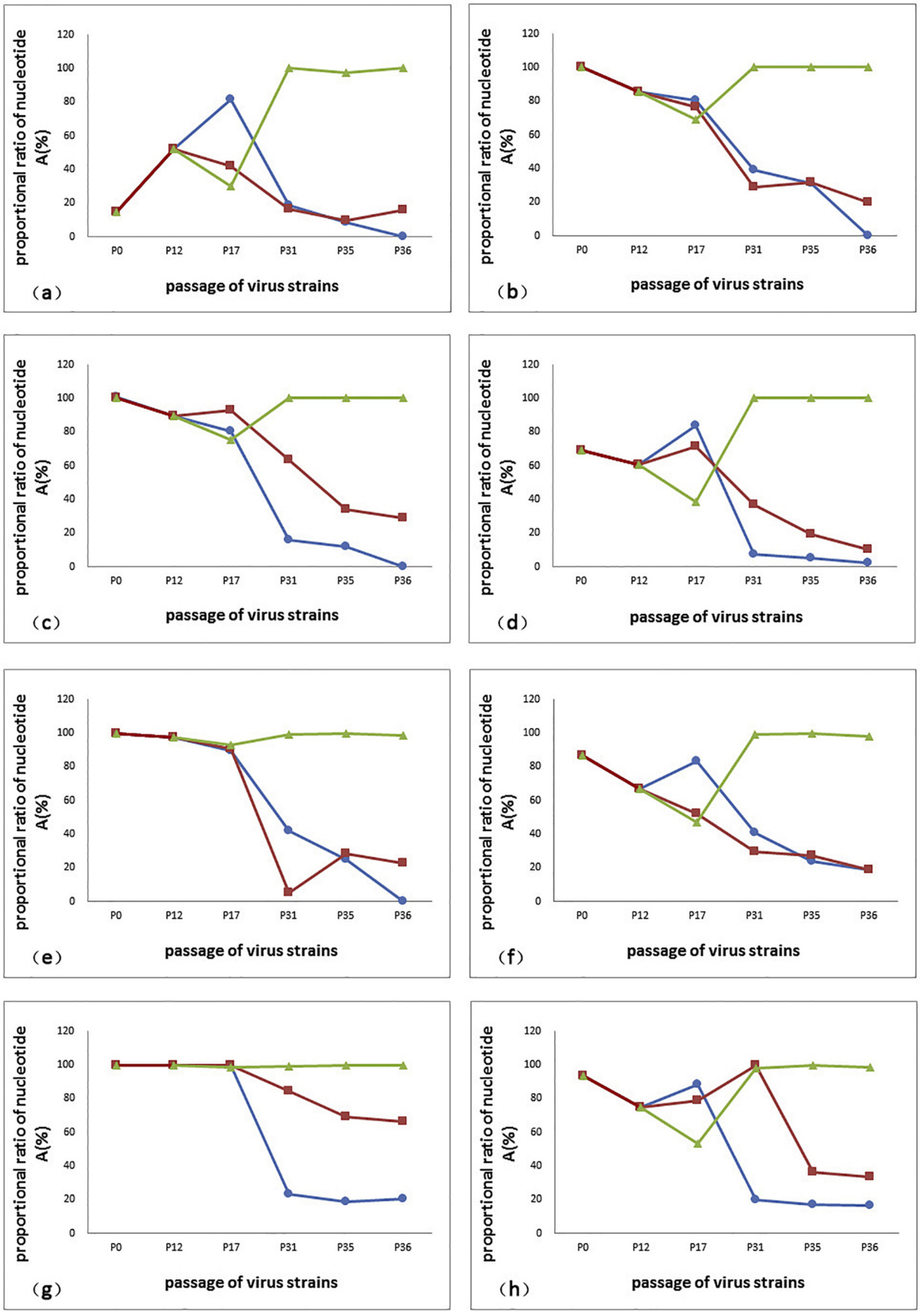

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Fig. 3. The proportional ratio of nucleotide A (%) at the mutation sites of VP1 during serial passage of virus strains at different temperatures for thermal adaption as following: clod-adapted (circle), cell-adapted (rectangle) and hot-adapted (triangle). (a) lineage #100, mutation site of A292G, by Sanger sequening. (b) lineage #100, mutation site of A434G, by Sanger sequencing. (c) lineage #101, mutation site of A292G, by Sanger sequencing. (d) lineage #101, mutation site of A434G, by Sanger sequencing. (e) lineage #100,mutation site of A355C/T, by NGS. (f) lineage #100, mutation site of A434G, by NGS. (g) lineage #101,mutation site of A355T, by NGS. (h) lineage #101, mutation site of A434G, by NGS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.10.012.

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