Attenuation of Human Enterovirus 71 High-Replication-Fidelity Variants in AG129 Mice

Tao Meng, Jimmy Kwang

ABSTRACT

In a screen for ribavirin resistance, a novel high-fidelity variant of human enterovirus 71 (EV71) with the single amino acid change L123F in its RNA-dependent RNA polymerase (RdRp or 3D) was identified. Based on the crystal structure of EV71 RdRp, L123 locates at the entrance of the RNA template binding channel, which might form a fidelity checkpoint. EV71 RdRp-L123F variants generated less progeny in a guanidine resistance assay and virus populations with lower mutation frequencies in cell culture passage due to their higher replication fidelity. However, compared with wild-type viruses, they did not show growth defects. In vivo infections further revealed that high-fidelity mutations L123F and G64R (previously reported) negatively impacted EV71 fitness and greatly reduced viral pathogenicity alone or together in AG129 mice. Interestingly, a variant with double mutations, RG/B4-G64R/L123F (where RG/B4 is an EV71 genotype B4 virus constructed by reverse genetics [RG]) showed higher fidelity in vitro and less virulence in vivo than any one of the above two single mutants. The 50% lethal dose (LD50) of the double mutant increased more than 500 times compared with the LD50 of wild-type RG/B4 in mice. The results indicated that these high-fidelity variants exhibited an attenuated pathogenic phenotype in vivo and offer promise as a live attenuated EV71 vaccine.

IMPORTANCE

The error-prone nature of the RNA-dependent RNA polymerase (RdRp) of RNA viruses during replication results in quasispecies and aids survival of virus populations under a wide range of selective pressures. Virus variants with higher replication fidelity exhibit lower genetic diversity and attenuated pathogenicity in vivo. Here, we identified a novel high-fidelity mutation L123F in the RdRp of human enterovirus 71 (EV71). We further elucidated that EV71 variants with the RdRp-L123F mutation and/or the previously identified high-fidelity mutation RdRp-G64R were attenuated in an AG129 mouse model. As EV71 has emerged as a serious worldwide health threat, especially in developing countries in the Asia-Pacific region, we urgently need EV71 vaccines. Learning from the poliovirus vaccination, we prefer live attenuated EV71 vaccines to inactivated EV71 vaccines in order to effectively control EV71 outbreaks at low cost. Our results imply a new means of attenuating EV71 and reducing its mutation rate at the same time.

RNA viruses are regarded by scientists as not simple, uniform organisms but genetically heterogeneous compositions in which each viral genome is likely to differ from every other virus by one or more point mutations (1, 2). The population with great genetic diversity thus generated, called quasispecies, facilitates the survival of the populations in the presence of selective pressures under which viruses containing advantageous adaptive mutations have better fitness to survive and produce progeny (3). The high replicative error rate of RNA viruses is caused by their error-prone RNA-dependent RNA polymerases (RdRp), with approximately one mistake made per 1,000 to 100,000 nucleotides copied (4–6).

Ribavirin, a guanosine nucleotide analog, is a mutagenic reagent that can be wrongly incorporated into RNA viral genomes during RNA synthesis because their RdRp lack proofreading and repair mechanisms. Ribavirin has been shown to increase error frequency and result in the accumulation of deleterious mutations which debilitate and even extinguish viral populations over several rounds of replication (6, 7). On the other hand, several RNA viruses with resistance to ribavirin have been identified and linked to an increased replication fidelity of their RdRp (8–10). As viral quasispecies are important to the evolution and adaptation of RNA viruses, the increased replication fidelity, which restricts the amplitude of quasispecies, results in an unfit or attenuated phenotype (9–13).

Human enterovirus 71 (EV71), like poliovirus, is a positive-strand RNA viral pathogen within the Picornaviridae family and Enterovirus genus. The genome of EV71 contains a single large coding region flanked by 5’ and 3’ untranslated regions (UTRs). The coding region is translated to a single polypeptide, which is then processed by viral proteases to yield four structural proteins (VP1 to VP4 in P1) and nonstructural proteins, including RdRp, which is usually named 3D (14, 15). Because of low replication fidelity, as with other RdRp, the RdRp of EV71 causes viral genetic diversity. Based on the VP1 gene sequence, EV71 is divided into three major genogroups (denoted A, B, and C) and various subgenogroups within genogroups B (B1 to B5) and C (C1 to C5) (16). Although most EV71 infections result in mild conditions such as hand-foot-and-mouth disease (HFMD), herpangina, or aseptic meningitis in young children, some cases are associated with severe neuroinfections further revealed that high-fidelity mutations L123F and G64R (previously reported) negatively impacted EV71 fitness and greatly reduced viral pathogenicity alone or together in AG129 mice. Interestingly, a variant with double mutations, RG/B4-G64R/L123F (where RG/B4 is an EV71 genotype B4 virus constructed by reverse genetics [RG]) showed higher fidelity in vitro and less virulence in vivo than any one of the above two single mutants. The 50% lethal dose (LD50) of the double mutant increased more than 500 times compared with the LD50 of wild-type RG/B4 in mice. The results indicated that these high-fidelity variants exhibited an attenuated pathogenic phenotype in vivo and offer promise as a live attenuated EV71 vaccine.
with severe neurological complications such as acute brainstem encephalitis and poliomylitis-like paralysis associated with high mortality during HFMD epidemics in the Asia-Pacific region (17, 18). Currently, there is no commercial antiviral therapy or vaccine against EV71 infection. Chemically inactivated EV71 grown in Vero cells is now used to produce vaccines for humans (19–21); however, live attenuated EV71 vaccines would be more effective to elicit both humoral and cellular immune responses at lower cost, as with control of other RNA viruses, such as influenza virus, poliovirus, measles viruses, rabies virus, rubella virus, and yellow fever virus (22–25).

As mouse is not a natural host of EV71, researchers have explored adapting EV71 in mouse brain or a mouse muscle line (26–28), but adapted EV71 viruses may present different characteristics, particularly in tropism-related features and clinical symptoms. Alternatively, immunodeficient mice (29, 30) and transgenic mice with a human EV71 receptor (31, 32) were found to be vulnerable to EV71 infection. The AG129 mouse is immunodeficient and without alpha/beta and gamma interferon (IFN) receptors; it can be infected by the non-mouse-adapted EV71 strain 41 (EV71-B4 in this paper, where B4 indicates the subgenogroup) (30). The infected mice display progressive limb paralysis prior to death and virus accumulation in the central nervous system, indicating a clear neurotropism of EV71 and making this model relevant for EV71 pathogenicity studies and for EV71 vaccines and drug testing.

Here, using a ribavirin resistance screen, we isolated a novel EV71 variant with the amino acid mutation L123F in its RdRp (RdRp-L123F) which increased viral replication fidelity without growth defects in rhabdomyosarcoma (RD) cells. EV71 variants with the L123F mutation had a lower mutation frequency in the presence or absence of ribavirin and produced fewer guanidine-resistant progeny in the presence of guanidine. RdRp-L123L locates at the entrance of the RNA template binding channel and might be involved in a hitherto unidentified fidelity checkpoint. We also generated a high-replication-fidelity variant, a subgenotype B4 virus constructed by reverse genetics ([RG] RG/B4-G64R) at another fidelity checkpoint, RdRp-G64, which has been previously described (8, 13, 33, 34), but RdRp-G64R caused slight growth defects in RD cells. In AG129 mice, the high-fidelity variants RG/B4-G64R and RG/B4-L123F and RG/B4-G64R/L123F with a double mutation showed less virulence and pathogenicity with lower viral titers and RNA copy numbers in mouse hind limb muscle and brain than wild-type RG/B4. These results suggested that high-replication-fidelity EV71 variants could be attenuated in vivo and offer a promising way to develop a live attenuated EV71 vaccine.

MATERIALS AND METHODS

Viruses, cells, and drugs. Wild-type EV71 strains EV71-B4 (5865/sin/000009, AF316321; subgenogroup B4) and EV71-B5 (NUH0083/SIN/08, FJ461781; subgenogroup B5) and reverse-genetics-generated EV71-C4 from synthesized genome (EU703812; subgenogroup C4) were used in this study (35). Human rhabdomyosarcoma cells (RD; ATCC number CCL-136), were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Biowest) and 1× Antibiotic-Antimycotic (Life Technologies). RD cells were maintained and infected with EV71 viruses at 37°C in a 5% CO2 incubator. Virus purification was carried out as reported previously (35). The following chemicals were obtained from Sigma-Aldrich: ribavirin (β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide, R9644-50MG), guanidine hydrochloride (G3272-500G), and l-glutamine (G3126-100G). Ribavirin 5′-phosphate and guanidine hydrochloride were dissolved in phosphate-buffered saline (PBS) at a concentration of 100 mM and stored at −20°C for long-term and at 4°C for short-term usage.

Ribavirin passages. Isolation of ribavirin-resistant variants with higher replicative fidelity was performed by serial passaging of EV71 subgenogroup B5 (EV71-B5) under gradually increasing concentrations of ribavirin. Briefly, 105 RD cells in 500 μl of DMEM were seeded into each well of a 24-well plate and incubated overnight. Next day, the RD cells were treated with ribavirin for 2 h and then infected by EV71-B5 at a multiplicity of infection (MOI) of <1 for 24 h. For each passage, virus was harvested by two freeze-thaw cycles and used in subsequent blind passages. In total, EV71-B5 was passed 20 times in the presence of 0.5 mM ribavirin, followed by 20 times with 1 mM and 20 times with 1.5 mM ribavirin. The viral populations from each passage were named by passage number, from B5 passage 1 (B5-P1) to B5-P60, and purified plaques were isolated from B5-P60 under the presence of 1.5 mM ribavirin in six-well plates by a standard plaque assay and designated B5-P60-C1 (where C1 indicates clone 1 isolated by plaque assay), B5-P60-C2, and so on. Briefly, six-well plates were seeded with RD cells at a density of 5 × 105 cells/well and grown overnight at 37°C. A total of 100 μl of 10-fold serial dilutions of virus was inoculated onto the RD cell monolayers. After incubation for 1 h at 37°C, the inoculum was removed, and the cells were washed with DMEM. Cells were then overlaid with 2 ml of DMEM with 5% FBS containing 0.8% agarose (A9045; Sigma) and incubated at 37°C in a 5% CO2 incubator. After 6 to 7 days of incubation, plaques containing viruses were selected using a 10-μl pipette tip to draw up the medium within the plaques.

Identification of molecular determinants of the ribavirin resistance variants. Viral RNA from EV71-B5, different virus passages, and six plaque-purified clones was purified by using an RNasey minikit (Qiagen). The RdRp gene was amplified by using SuperScript III one-step reverse transcription-PCR (RT-PCR) with Platinum Taq polymerase (Life technologies) and two primers: UniEV71-RdRp-f and UniEV71-r (Table 1). The RdRp cDNA was extracted from the gel using a QIAquick gel extraction kit (Qiagen) and then inserted into pGEMT vector (Promega) for sequencing. The RdRp gene and corresponding protein sequences of variants were analyzed using the Lasergene software package (DNASTAR)
and Chromas (Technelysium Pty., Ltd.). The positions of mutations on the EV71 RdRp protein were located on its crystal structure (36).

Construction of infectious plasmids of EV71 and generation of virus stocks. The genomes of EV71-B4 and EV71-B5 wild-type viruses were first amplified by RT-PCR and put under human RNA polymerase I promoter reverse genetics (RG) system as described earlier (37), and the infectious plasmids were named pJE-T-B4 and pJE-T-B5, respectively. The infectious plasmid pJE-T-C4 containing synthetic cDNA of EV71-C4 has been previously described (35). The mutations G64R and L123F in the RdRp gene were introduced into the plasmids pJE-T-B4, pJE-T-B5, and pJE-T-C4 by site-directed mutagenesis with corresponding primers, listed in Table 1. The infectious viruses RG/B4, RG/B5, RG/C4, RG/B4-G64R, RG/B4-L123F, RG/B5-L123F, RG/C4-L123F, and RG/B4-G64R/L123F were generated by direct transfection of their corresponding infectious plasmids into RD cells. All generated viruses were further propagated in RD cells for four passages, and the correctness of their RdRp genes of the fourth passage was confirmed by sequencing. All following cell and animal infection works used fourth-passage RG viruses unless indicated otherwise.

Quantification of virus by TCID50 assay and SBGR qRT-PCR. RD cells were seeded into a 96-well plate at 10^4 cells in 100 μl of DMEM with 5% FBS per well. Four hours later, 100 μl of 10-fold serial dilutions of virus in DMEM with 5% FBS was transferred into each well in the plate. After 5 days, infected wells were counted for clear cytopathic effect (CPE) on cell monolayers. Values of the 50% tissue culture infectious doses (TCID50) were determined by the Reed and Muench method. Viral RNA genome in infected cells and mouse tissues was purified by using an RNeasy minikit (Qiagen), and the copy numbers were determined by SYBR green (SBGR) quantitative RT-PCR (qRT-PCR). Briefly, primers EV-5UTR-f(445) and EV-5UTR-r(557) and a QuantiFast SYBR green RT-PCR kit (Qiagen) were used to detect EV71 RNA. The RT-PCR thermal cycling conditions were applied at an initial incubation at 50°C for 10 min (reverse transcription) and at 95°C for 5 min (initial PCR activation step), followed by 35 cycles of 95°C for 10 s (denaturation), 60°C for 30 s (annealing and extension), and signal collection. Melting-curve data were collected from 50 to 95°C at a ramping rate of 1°C/5 s. The reaction was carried out using a Rotor-Gene Q real-time PCR cycler (Qiagen). A standard curve of copy numbers and corresponding threshold cycle (Ct) values was plotted using pJE-T-B4 plasmid, and the relative expression levels of viral genomes were normalized to the expression value of mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Replication kinetics. For replication studies of RG viruses, RD monolayers at a density of 2 × 10^5 cells per well in 24-well plates were either pretreated with 0.5 mM, 1 mM, 1.5 mM, or 2 mM ribavirin or mock treated and infected at an MOI of 1. For one-step growth kinetics, infected RD cells were frozen at different time points postinfection, and viruses were titrated by TCID50 assay after three freeze-thaw cycles and centrifugation at 3,000 × g for 10 min. All experiments were carried out in triplicate, and the titration was duplicated for each experiment.

Guandine resistance assay of RG/B4 and RG/B4-L123F. The guandine sensitivities of plaque-purified RG/B4-L123F populations were compared to those of the plaque-purified RG/B4 populations with growth (0.5 mM) or without guandine for 24 h. Briefly, six plaque-purified populations of both RG/B4 and RG/B4-L123F were selected using plaque assays as described above and propagated in RD cells twice to the titers of more than 10^6 TCID50/ml. A total of 2 × 10^3 RD cells per well in 24-well plates were either untreated or treated with 0.5 mM guandine hydrochloride for 2 h at 37°C incubator. Cells were infected at an MOI of 1 and incubated for another 24 h. Viruses were released from cells by the freeze-thaw method, and the viral titers in supernatant of the lysate was determined by TCID50 assay.

Determination of mutation frequency of RG/B4 and RG/B4-L123F. RG/B4 and RG/B4-L123F were serially passaged in RD cells at an MOI of 1 in the presence or absence of 1 mM ribavirin as described above. Viral RNA from 12th-passage virus stocks was extracted, and cDNAs were synthesized by a RevertAid first-strand cDNA synthesis kit (Fermentas) using oligo(dT)12 primer. The P1 gene was amplified by Pfu Ultra II Hotstart PCR Master Mix (Agilent Technologies) using primers B4-P1-f and B4-P1-r, and the RdRp gene was amplified using UniEV71-RdRp-f and UniEV71-r. The PCR products were inserted into pJE-T1.2 vector (Fermentas) for sequencing according to the procedure of the kit. For each virus population, eight P1 and eight RdRp gene sequences were obtained and analyzed using the Lasergene software package (DNASTAR). The number of mutations per 10^6 nucleotides was determined using total mutations identified per population over the total number of nucleotides sequenced for that population multiplied by 10^6. If the same mutation recurred in the same population, it was counted only once.

Mouse infection. AG129 mice were obtained from B&K Universal (United Kingdom) and housed in individual ventilated cages inside a biosafety level 3 (BSL3) lab for animal care and use. All animal experiments were carried out in accordance with the Guides for Animal Experiments of the National Institute of Infectious Diseases (NIID), and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Temasek Life Sciences Laboratory, Ltd., Singapore (IACUC project approval no. TLL-13-002, Increased Fidelity Reduces Human Enterovirus 71 Fitness and Virulence in AG129 Mice).

Ten-day-old AG129 neonates were administered with 0.1 ml of PBS containing 10^4 to 10^5 TCID50 of EV71 via the intraperitoneal (i.p.) route. The clinical scores and survival rates were recorded daily until 21 days postinfection (p.i.). For viral propagation and distribution in the infected mice, hind leg muscle and brain were harvested, weighed, and stored at −80°C after euthanasia of mice with CO2. Samples were homogenized by using a TissueLyser LT homogenizer (Qiagen) in DMEM with 10% FBS, l-glutamine, and 10% Antibiotic-Antimycotic. The virus titers in the supernatants of clarified homogenates (3,000 × g for 10 min at 4°C) were determined by TCID50 assay. Each assay was carried out in triplicate. The RNAs from homogenates were extracted as described above, and viral RNA copy numbers per gram of tissue were determined by SBGR qRT-PCR.

Statistics. All statistical analyses were performed using Student’s unpaired t test. A P value of <0.05 was considered statistically significant. The mean values and 1 standard deviation (mean ± SD) are shown unless specifically indicated otherwise.

RESULTS

Generation of ribavirin-resistant EV71-B5 variants. In order to generate EV71-B5 variants with ribavirin resistance, we performed serial passages of the virus in the presence of ribavirin with a concentration from 0.5 mM to 1.5 mM as described in Materials and Methods. The total of 60 passages finally generated a B5-P60 viral population which showed ribavirin resistance even at a very high concentration of ribavirin but without a significant growth defect. As shown in Fig. 1A, the titer of the B5-P60 population reached an average of 10^5.5 TCID50/ml after 24 h with infection at an MOI of 1 in RD cells without ribavirin, while the parental B5 virus was capable of growing only to an average titer of 10^8.2 TCID50/ml. The virus titer ratio results (Fig. 1B) obviously showed that the growth titers of B5-P60 in the presence of 1 mM and 2 mM ribavirin were approximately 23% and 7.5% of that in the absence of ribavirin. In contrast, the growth of B5 was significantly inhibited in the presence of 1 mM and 2 mM ribavirin, and the titers were 3% and 1.6% of those in the absence of ribavirin, respectively. Thus, the results suggested an increase in the capacity to produce progeny in the presence of ribavirin for B5-P60 compared with B5. Furthermore, one plaque-purified clone, B5-P60-C1, isolated from the B5-P60 population showed more ribavirin resistance in the growth profile, and its growth titers were as high...
notype. (A) RD cell monolayers were infected at an MOI of 1 TCID₅₀/cell with different viral populations in the absence or presence of ribavirin (1 mM or 2 mM). The progeny viruses were titrated at 24 h postinfection. The experiment was carried out in triplicate, and means ± SDs are shown. (B) Ratios of mean titers of progeny viruses produced in the presence of ribavirin (R⁻) to those produced in the absence of ribavirin (R⁺) showed that populations B5-P60 and B5-P60-C1 produced more progeny than the parental B5 virus in the presence of ribavirin and, hence, had acquired ribavirin resistance.

Identification of the molecular determinants for EV71 resistance to ribavirin. To identify the mutation or mutations responsible for ribavirin resistance, RdRp genes of EV71-B5, B5-P40, B5-P60, and plaque-purified clones including B5-P60-C1 were amplified, sequenced, and compared. As shown in Table 2, ribavirin resulted in accumulation of G-to-A and C-to-T transition mutations. The RdRp gene of B5-P60-C1 contained many silent mutations and one nucleotide substitution, C367T, causing an amino acid change from leucine to phenylalanine at position 123 of RdRp, L123F, compared to the RdRp gene of the parental EV71-B5. RdRp-L123F was found in a minority of the RdRp gene sequences of the B5-P40 population but in a majority of the B5-P60 population based on gene sequencing chromatograms (Fig. 2). This result indicated that L123F is just one possible mutation that confers ribavirin resistance on the B5-P60 population. Apparently, other ribavirin-resistant virus progeny bearing additional amino acid substitutions were generated during passages.

To test whether a single amino acid mutation, RdRp-L123F, was sufficient to confer ribavirin resistance on EV71, three infectious viral cDNA constructs and their corresponding mutants with RdRp-L123F were generated using a human RNA polymerase I promoter-driven reverse genetics (RG) system (37). The generated viruses RG/B4, RG/B5, RG/C4, RG/B4-L123F, RG/B5-L123F, and RG/C4-L123F belong to the three subgenogroups B4, B5, and C4 and were grown in RD cells under the absence or presence of 2 mM ribavirin. Although the three types of EV71 had different growth profiles (Fig. 3A), their L123F variants showed ribavirin resistance as the titers of their progeny decreased less than those of the parental viruses in the presence of ribavirin. For example, the growth titers of RG/B4, RG/B5, and RG/C4 in the presence of 2 mM ribavirin were 1.2%, 1.9%, and 3.9%, respectively, of that in the absence of ribavirin, while the growth titers of RG/B4-L123F, RG/B5-L123F, and RG/C4-L123F in the presence of ribavirin decreased less to 17.8%, 9%, and 14.7%, respectively, of that in the absence of ribavirin (Fig. 3B); that is, the capacity of resisting ribavirin and producing progeny increased 14-, 4-, and 3-fold for the three L123F variants, respectively.

We further compared the replicative kinetics of RG/B4 and RG/B4-L123F in the presence or absence of ribavirin. With the concentration of ribavirin increasing from 0.5 mM to 2 mM in RD cell culture medium, RG/B4-L123F consistently replicated to significantly higher titers than RG/B4 after 20 h of infection at an MOI of 1 (Fig. 3C). To determine whether the higher RG/B4-L123F titer in the presence of ribavirin was due to increased replicative capacity, one-step growth analysis of RG/B4 and RG/B4-L123F in the absence of ribavirin was performed. Their one-step growth curves were not significantly different, indicating that the mutation L123F did not significantly impact virus multiplication (Fig. 3D). In the presence of 2 mM ribavirin, differences in the growth curves of RG/B4 and RG/B4-L123F were observed. At each time point after infection, the titer of RG/B4-L123F was significantly higher than that of RG/B4; that is, RG/B4-L123F was resistant to ribavirin. The best resistance performance of RG/B4-L123F was at 20 h postinfection; the progeny titer of RG/B4-L123F in the presence of ribavirin was 10⁵.₃₈ TCID₅₀/ml compared to 10⁵.₃₇ TCID₅₀/ml in the absence of ribavirin, while the progeny titer of RG/B4 plummeted from 10⁷.₅₆ TCID₅₀/ml in the absence of ribavirin to 10⁷.₃₅ TCID₅₀/ml in the presence of ribavirin (Fig. 3D).

All of the above results substantiated that one amino acid mutation, L123F in RdRp, was sufficient to confer ribavirin resistance on EV71 in vitro.

### Table 2: Mutations present in the RdRp gene of ribavirin-resistant B5-P60-C1 compared with the parental EV71-B5

<table>
<thead>
<tr>
<th>Nucleotide position in the RdRp gene</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
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<tr>
<td>63</td>
<td>T→C</td>
<td>None</td>
</tr>
<tr>
<td>141</td>
<td>C→T</td>
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<tr>
<td>180</td>
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<td>300</td>
<td>G→A</td>
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<td>C→T</td>
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<tr>
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<td>603</td>
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<tr>
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L123 is conserved and located at the RNA template binding channel of EV71 RdRp. To examine whether RdRp-L123 is functionally important in viral replication and to understand the structural mechanism for ribavirin resistance of L123F variants, we first performed protein sequence alignment of the RdRp of viruses in the Picornaviridae family and then located L123 in the crystal structure of EV71 RdRp (36). The alignment results (Fig. 4A) indicated that L123 is identical for all published EV71 and CVA16 sequences in the NCBI database and highly conserved in other enteroviruses, such as coxsackie B3 virus (CVB3), echovirus, human rhinoviruses (HRVs), and poliovirus type 1 (PV1), and other picornaviruses, such as Aichi virus (AiV) and encephalo-
Increased fidelity mutations of EV71 RdRp. To test the possibility that the ribavirin resistance of L123F variants results from the increased replication fidelity of RdRp, as previously reported in poliovirus (8), a guanidine resistance assay and mutation frequency test of RG/B4 and RG/B4-L123F were performed. EV71 resistance to guanidine can be generated by several amino acid substitutions in the 2C coding region (38). From the rate of random incorporation of guanidine resistance mutations in 2C, the replication fidelity of RG/B4 and RG/B4-L123F was estimated. Six plaque-purified isolates from both RG/B4 and RG/B4-L123F viruses independently infected RD cells at an MOI of 1 for 24 h in the presence or absence of 0.5 mM guanidine hydrochloride (Fig. 5A). The guanidine resistance frequency of RG/B4-L123F (0.0017) was apparently 6-fold lower than that of RG/B4 (0.01) on average although variation existed between individual isolates (Fig. 5B). Predictably, RG/B4-L123F produced lower progeny titers under guanidine pressure because fewer guanidine resistance mutations in 2C were generated due to its higher replication fidelity than RG/B4.

Next, the average mutation frequencies of RG/B4 and RG/B4-L123F were determined by direct sequencing of genomic cDNA of viral populations after 12 passages in the absence or presence of 0.5 mM ribavirin (Table 3). Eight P1 gene and eight RdRp gene sequences (total of 31,872 nucleotides) were analyzed for each population. The results indicated that RG/B4 was highly diverse, having on average 3.45 and 16.93 mutations per 10⁴ nucleotides in the absence and presence of ribavirin, respectively, while RG/B4-L123F restricted the viral population diversity to only 1.88 and 4.39 mutations per 10⁴ nucleotides in the absence and presence of ribavirin, respectively. On the other hand, when the mutagen ribavirin was added, the mutation frequency of RG/B4 increased 3.91 times (from 3.45 to 16.93 mutations per 10⁴ nucleotides), while the mutation frequency of RG/B4-L123F increased only 1.33 times (from 1.88 to 4.39 mutations per 10⁴ nucleotides). Therefore, the RG/B4-L123F virus had a lower mutation frequency and less genetic diversity than RG/B4 over 12 passages because of its higher replication fidelity. Moreover, the sequences of the RdRp gene of RG/B4-L123F indicated that the L123F mutation was genetically stable after 12 passages in the presence or absence of ribavirin.

The above results showed that the replication fidelity of EV71 can be improved by a single amino acid mutation, L123F, in its RdRp.

**Attenuation of high-replication-fidelity EV71 variants in AG129 mice.** Previous studies revealed that high-replication-fidelity RNA virus variants are attenuated through restricted replication and lower fitness in an animal model (10–13). To evaluate the effect of high-replication-fidelity EV71 variants on viral pathogenicity in vivo, we used the AG129 mouse model, which has been well defined for wild-type EV71-B4 infection with neurotropism (30). We also generated RB4/L123F variants with mutations at the position G64 of RdRp, which was identified as participating in a fidelity checkpoint in the RdRp of poliovirus (13). Similar to the findings of a previous report (33), most of these variants were lethal or had a severe defect in growth (data not shown). Only RB4/G64R, RB4/G64R/L123F was stable, but its growth was slightly attenuated compared to that of RB4/B4 (Fig. 6A). We further generated a double mutant, RB4/G64R/L123F to evaluate whether the high-fidelity mutations G64R and L123F can synergize to boost EV71 fidelity. The double mutant RB4/G64R/L123F did not further reduce viral growth titers compared with RB4/G64R. Both RB4/G64R and RB4/G64R/L123F displayed high replication fidelity as they resisted ribavirin and generated less guanidine-resistant progeny than wild-type RB4/B4 (Fig. 6B and C); moreover, their mutation frequencies decreased in the presence or
absence of 0.5 mM ribavirin after 12 passages in RD cells compared with RG/B4 (Table 3). Interestingly, the double mutant RG/B4-G64R/L123F had the lowest reduction in the titer of its progeny among all high-fidelity variants in the presence of ribavirin (Fig. 6B), and its mutation frequency (2.82) was significantly lower than that of RG/B4-G64R (5.33) and of RG/B4-L123F (4.39) after passages in RD cells with ribavirin (Table 3). Therefore, RG/B4-G64R/L123F had higher fidelity than RG/B4-G64R or RG/B4-L123F, indicating that G64R and L123F cooperatively boosted the fidelity of EV71-B4.

Ten-day-old AG129 mice were intraperitoneally inoculated in parallel with serial dilutions (10⁹ to 10⁵ TCID₅₀ per mouse) of viruses RG/B4, RG/B4-G64R, RG/B4-L123F, and RG/B4-G64R/L123F. We initially observed the survival rate and determined the 50% lethal dose (LD₅₀) for each virus (Fig. 7 and Table 4). After inoculation, the wild-type RG/B4 quickly invaded the central nervous system, resulting in paralysis and death. In comparison, all high-fidelity variants, RG/B4-G64R, RG/B4-L123F, and RG/B4-G64R/L123F, were less virulent and had delayed onset of symptoms. For example, at the virus dose of 10⁷ TCID₅₀, all of the mice infected with RG/B4 died between day 5 and day 12 postinfection, while mice infected with RG/B4-L123F started to die only at day 7 postinfection, and only 37.5% of mice died. For all the mice infected with RG/B4-G64R or RG/B4-G64R/L123F, none of them showed paralysis or died throughout the whole experiment (Fig. 7). The LD₅₀ of RG/B4 is 4.3 × 10⁵ TCID₅₀, while LD₅₀ values of

### TABLE 3

<table>
<thead>
<tr>
<th>Condition and virus</th>
<th>Mutation</th>
<th>A→C</th>
<th>A→G</th>
<th>T→C</th>
<th>T→G</th>
<th>C→T</th>
<th>G→A</th>
<th>Total no. of mutations</th>
<th>No. of mutations per 10⁴ nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribavirin treatment</td>
<td>RG/B4</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>25</td>
<td>17</td>
<td>54</td>
<td>16.93</td>
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<td></td>
<td>RG/B4-G64R</td>
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<td>1</td>
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<td>5</td>
<td>17</td>
<td>14</td>
<td>17</td>
<td>5.33</td>
</tr>
<tr>
<td></td>
<td>RG/B4-L123F</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>5</td>
<td>14</td>
<td>9</td>
<td>9</td>
<td>4.39</td>
</tr>
<tr>
<td></td>
<td>RG/B4-G64R/L123F</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>9</td>
<td>2.82</td>
<td>2.82</td>
<td>2.82</td>
<td>2.82</td>
</tr>
<tr>
<td>Untreated</td>
<td>RG/B4</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>11</td>
<td>3.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RG/B4-G64R</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>1.88</td>
<td>1.88</td>
<td>1.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RG/B4-L123F</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>1.88</td>
<td>1.88</td>
<td>1.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RG/B4-G64R/L123F</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>1.57</td>
<td>1.57</td>
<td>1.57</td>
<td>1.57</td>
</tr>
</tbody>
</table>

*Eight P1 gene (2586 nucleotides each) and eight RdRp gene (1398 nucleotides each) sequences (total of 31,872 nucleotides) were analyzed for each population. The results indicated that RG/B4 was highly diverse, having on average 3.45 and 16.93 mutations per 10⁴ nucleotides in the absence and presence of ribavirin, respectively, while all high-fidelity variants had fewer mutations per 10⁴ nucleotides in the absence and presence of ribavirin.

*The double mutant RG/B4-G64R/L123F had a significantly lower mutation frequency (2.82) than RG/B4-G64R (5.33) and RG/B4-L123F (4.39) per 10⁴ nucleotides in the presence of ribavirin, indicating that the fidelity of EV71 was further elevated by two mutations, G64R and L123F, in its RdRp.
the high-fidelity variants RG/B4-G64R, RG/B4-L123F, and RG/B4-G64R/L123F are 4.0 \times 10^7, 1.6 \times 10^7, and 2.3 \times 10^8 TCID_{50}, respectively, with 93, 37, and 535 times the LD_{50} of RG/B4, respectively (Table 4).

We next assessed whether high-replication-fidelity EV71 variants influence the kinetics and severity of virus infection in mice. The hind limb muscle and brains (two primary targets of EV71 infection) of infected mice were harvested at days 4, 8, and 12 postinfection after inoculation of each variant at a dose of 10^7 TCID_{50} per mouse. The viral titers and RNA copy numbers were

FIG 6 Growth profile of high-fidelity variants RG/B4-G64R, RG/B4-L123F, and RG/B4-G64R/L123F. (A) One-step growth curves of viruses. The growth titer of RG/B4-G64R is significantly lower than that of RG/B4 (*, \( P < 0.01 \); **, \( P < 0.001 \); \( n = 6 \)), while there is no significant difference between the growth titers of the parental RG/B4 and RG/B4-L123F or between the growth titers of RG/B4-G64R and RG/B4-G64R/L123F. (B) Ratios of titers of progeny viruses produced in the presence of 2 mM ribavirin to those produced in the absence of ribavirin after 20 h of infection. All high-fidelity variants produced more progeny viruses than the parental RG/B4 (*, \( P < 0.001 \); \( n = 6 \)). (C) Guanidine resistance frequency assay. All high-fidelity variants had lower guanidine resistant frequencies than RG/B4 due to their lower capacity of generating guanidine-resistant mutations (*, \( P < 0.01 \); **, \( P < 0.001 \); \( n = 6 \)). All experiments were carried out in triplicate, and the titration was duplicated for each experiment. Means ± SDs are shown.
calculated (Fig. 8A and B). The results indicated that RG/B4 accumulated in the hind limb muscle and brain throughout the whole experiment; the viral titer and RNA copy number reached the highest levels before the death of infected mice, similarly to a previous description of wild-type B4 infection in AG129 mice (30). In contrast, all high-replication-fidelity variants showed lower viral titers and RNA copy numbers in the hind limb muscle and brain than RG/B4 at each time point. Interestingly, the viral titers and RNA copy numbers of all high-fidelity variants declined at later days postinfection. Therefore, the high-replication-fidelity variants showed restricted viral replication and low fitness in AG129 mice; that is, they were attenuated in vivo. We also amplified and sequenced RdRp genes of these high-fidelity variants in the muscle and brain of the infected mice at the 12th day postinfection. The sequencing results substantiated that the high-fidelity mutations G64R and L123F, alone or together, were genetically stable in the

![Survival rates of AG129 mice infected by RG/B4 and its high-fidelity variants. Ten-day-old AG129 mice were intraperitoneally inoculated with serially diluted doses of EV71 ranging from $10^5$ to $10^9$ TCID$_{50}$ per mouse. Survival rates for each virus were monitored on a daily basis, and all dead mice showed clear hind limb paralysis 1 to 2 days prior to their death. Each group contained at least 6 mice (detailed numbers are given in Table 4). High-fidelity variants RG/B4-G64R, RG/B4-L123F, and RG/B4-G64R/L123F were less virulent than RG/B4 and show delayed onset of symptoms at even the highest doses.](http://jvi.asm.org/)

### Table 4 Comparison of LD$_{50}$s of high-fidelity variants and parental RG/B4

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus amt (TCID$_{50}$)</th>
<th>Mouse no.</th>
<th>Death rate ( %)</th>
<th>LD$<em>{50}$ (TCID$</em>{50}$)</th>
<th>Ratio of LD$<em>{50}$ to the LD$</em>{50}$ of RG/B4</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG/B4</td>
<td>$10^8$</td>
<td>6</td>
<td>100</td>
<td>$4.3 \times 10^3$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$10^7$</td>
<td>8</td>
<td>100</td>
<td>$1.6 \times 10^2$</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>$10^6$</td>
<td>9</td>
<td>78 (7/9)</td>
<td>$10^2$</td>
<td>60</td>
</tr>
<tr>
<td>RG/B4-L123F</td>
<td>$10^8$</td>
<td>6</td>
<td>100</td>
<td>$4 \times 10^7$</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>$10^7$</td>
<td>8</td>
<td>37.5 (3/8)</td>
<td>$10^7$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>$10^6$</td>
<td>6</td>
<td>0</td>
<td>$10^3$</td>
<td>60</td>
</tr>
<tr>
<td>RG/B4-G64R</td>
<td>$10^9$</td>
<td>7</td>
<td>100</td>
<td>$9 \times 10^7$</td>
<td>535</td>
</tr>
<tr>
<td></td>
<td>$10^8$</td>
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<td>83 (5/6)</td>
<td>$10^7$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>$10^7$</td>
<td>6</td>
<td>0</td>
<td>$10^9$</td>
<td>60</td>
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<tr>
<td>RG/B4-G64R/L123F</td>
<td>$10^9$</td>
<td>6</td>
<td>83 (5/6)</td>
<td>$10^7$</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>$10^8$</td>
<td>6</td>
<td>33 (2/6)</td>
<td>$10^7$</td>
<td>60</td>
</tr>
</tbody>
</table>

*LD$_{50}$ of all high-fidelity variants were significantly higher than the LD$_{50}$ of the parental RG/B4.

*Values in parentheses are the number of mice that died/total number of mice in the group.*
infected mice and implied that these high-fidelity variants are promising vaccine candidates.

**DISCUSSION**

In this report, we identified a novel mutation, L123F, in the RdRp of EV71 through ribavirin resistance screening and demonstrated that this mutant has higher replication fidelity than the wild type. The ribavirin resistance of EV71 could be explained by several hypothetical mechanisms. First, the L123F variants could simply replicate at a lower rate and therefore acquire fewer lethal mutations and avoid “error catastrophe” over any given time period. The fact that the RdRp-L123F mutation did not cause growth defects can exclude this hypothesis. The second probable mechanism could be that RdRp-L123F reduces the binding affinity to ribavirin and decreases the chance of misincorporation of ribavirin into EV71 genomes (39). However, the crystal structure of EV71 RdRp reveals that the location of L123 excludes the possible interaction between L123 and ribavirin (Fig. 4), and, more, the RdRp-L123F variants resisted another drug as well, guanidine, which directly targets 2C but not RdRp (38). Therefore, we prefer the third mechanism, i.e., that RdRp-L123F confers a general increase in RNA replication fidelity. As RdRp-L123 locates in the entrance of the RNA template binding channel, the mutation L123F may alter the conformational structure and interaction of the RdRp and viral RNA complex, and this alteration could confer higher replication fidelity on RdRp. Moreover, the result that the mutation frequency of RG/B4-L123F was much lower than that of RG/B4 over time in the presence or absence of ribavirin substantiated that RdRp-L123F overall had higher replication fidelity (Table 3).

High-replication-fidelity variants have been described in EV71 (33, 34) and other RNA viruses (8–10); however, these mutations are in different regions of the viral RdRp. Therefore, RNA viruses have different fidelity checkpoints consisting of multiple residues which may work alone or cooperatively. It is well known that the RdRp-G64 of poliovirus cooperates with G1, A239, and L241 to form a hydrogen bond network which determines the spatial positioning of D238. And the D238 interacts with the 2’ OH group of the incoming nucleotide into the catalytic site of polymerase (40, 41). Therefore, RdRp-G64 indirectly influences viral RNA polymerization of poliovirus. A comparison of the RdRp crystal structures between poliovirus and EV71 revealed that the RdRp-G64 of EV71 might have the same function as the RdRp-G64 of poliovirus (36), and a previous report (33) and our results substantiated that RdRp-G64 is a fidelity checkpoint as well in EV71. It is impossible for the RdRp-L123 of EV71 to be involved in RNA polymerization catalysis during viral replication because it is very far from the catalytic site. However, L123 locates in the entrance of the RNA template binding channel, which allows viral genomic RNA template to enter and then stabilize it during viral replication.

**FIG 8** Viral titers and RNA copy numbers in organs from infected AG129 mice. (A) Ten-day-old mice were intraperitoneally inoculated with 10^7 TCID_{50} per mouse. At days 4, 8, and 12 postinfection, mice (n = 6) were euthanized (#, 2 moribund mice euthanized at day 7; ##, 4 moribund mice euthanized at day 11), and the viral titers per gram of hind limb muscle (●) and brains (○) were determined by a TCID_{50} assay whose minimum detection limit was 10^2 TCID_{50}/gram so that viral titers were postulated as 10^2 TCID_{50}/gram if they could not be determined. Solid lines and dashed lines indicate the average values of viral titers in muscle and brains, respectively. (B) Viral RNA copy numbers in hind limb muscle and brains from infected mice determined by SBGR quantitative RT-PCR. The viral titers and RNA copy numbers in mice infected with high-fidelity variants were significantly lower than those in mice infected with wild-type RG/B4 at each time point (*, P < 0.05; **, P < 0.01; ***, P < 0.001; n = 6).
(36). The mutation L123F could cause the conformational change of RdRp before and/or during RNA synthesis to confer ribavirin resistance on EV71. Therefore, we predicted that RdRp-L123 could be involved in a hitherto unidentified fidelity checkpoint in EV71, but the structural details need further study.

Although RdRp-L123 is highly conserved in some picornaviruses (Fig. 4), the mutation RdRp-L123F may not generate the same growth and high-replication-fidelity profiles in these picornaviruses as in EV71. Similar results have already been described in mutants at the position RdRp-G64 of poliovirus (13), CVB3 (42), and EV71 (33). The same mutations which confer high fidelity on poliovirus were nonviable, growth defective, unstable, or even had decreased replication fidelity in CVB3 and EV71. The possible reason could be the subtle structural differences between their RdRp and/or changes in interactions of the RdRp mutants and their corresponding RNA genome complex during virus replication. Therefore, the best way to discover high-fidelity variants is RNA virus mutation (such as ribavirin) resistance screening. And the direct engineering of residues in fidelity checkpoints in the viral RdRp is also promising if the structure of RdRp is well defined (13, 42).

Mutation frequency can be very high in the presence of a low-fidelity RdRp. In a complex environment, the ability to generate a quasispecies may allow virus populations to respond and adapt fast (43–46). Fidelity variants with wild-type-like replication kinetics are valuable tools to understand the roles of genetic diversity and mutation frequency in viral fitness and to evaluate the antiviral treatments (47). Indeed, the RdRp of an RNA virus is a quite elaborate machine coordinating viral replication fidelity and fitness under various pressures. Higher-fidelity variants may become extinct when facing host environmental changes, immune responses, transmission bottlenecks, and so on (10, 11, 13, 48), while much lower fidelity variants may also be restricted and extinguished soon due to higher lethal mutation frequency (42). The correlation between replication fidelity and fitness in vivo was first demonstrated with higher-fidelity polioviruses (11–13) and later with higher-fidelity chikungunya virus (CHIKV) (10) and lower-fidelity coxsackievirus B3 (CVB3) (42). Both higher- and lower-fidelity variants have less fitness and are attenuated in vivo.

Although the mouse-adapted EV71 strain MP-26M with high-fidelity mutations did not show dramatically lower virulence in BALB/c mice (34), both RdRp-G64R and RdRp-L123F significantly reduced the pathogenicity of EV71-B4 in the AG129 mouse model alone or together according to the \(LD_{50}\) test and amount of viral RNA in the infected tissues. The reasons for the discrepancy between the two experiments could be due to different virus strains and mouse models. For example, the virulence of MP-26M in BALB/c mice may not depend mainly on its ability to generate diverse populations for fitness due to the low fidelity of its RdRp but on specific mutations which accumulated after passages in mouse brain. Therefore, the increase in replication fidelity of MP-26M failed to reduce its virulence in mice. In contrast, the virulence of wild-type EV71-B4 and of its high-fidelity variants in mice depends on their mutant abilities, which result in replication-efficient mutations in different tissues and organs, especially in brain. The EV71-B4 high-fidelity variants exhibited low pathogenicity because they were unable to generate these replication-efficient mutations in time.

Interestingly, the double mutant RG/B4-G64R/L123F was attenuated in vivo (Table 4) in comparison with RG/B4-G64R or RG/B4-L123F, which coincides with the result that the fidelity of RG/B4-G64R/L123F was higher than that of RG/B4-G64R or RG/B4-L123F (Fig. 6B and Table 3). Therefore, it seems that a negative correlation exists between the fidelity and virulence of EV71-B4 in AG129 mice. The higher-fidelity variants exhibited lower genetic diversity, which probably reduced the viral capacity to generate mutants favoring viral growth and dissemination (10–13). As reported in this paper, the viral titers and RNA copy numbers in hind limb muscle and brain of mice infected with RG/B4-G64R/L123F were much lower than those of mice infected with RG/B4, RG/B4-G64R, or RG/B4-L123F (Fig. 8).

One important issue about the relationship between variants with increased fidelity and their attenuation in vivo is whether or not these mutations in RdRp cause growth defects as growth defects also result in decreased virulence. Although RG/B4-L123F did not show growth defects while RG/B4-G64R and RG/B4-G64R/L123F did in RD cell culture, it is necessary to compare these viruses with the wild-type RG/B4 through competitive growth in mice in order to determine whether subtle growth defects happen in vivo (11). It also has scientific merit to evaluate which feature, the growth defects or enhanced fidelity of these variants, played a major role in their attenuation in mice in further experiments. As high-fidelity variants result in limited genomic diversity which negatively affects viral pathogenesis, expanding the diversity of these variants by chemical mutagenesis, such as through ribavirin, prior to infection may restore their pathogenicity in mice (12), and this could be a way to determine whether increased fidelity truly contributes to the attenuation of these high-fidelity variants in vivo.

Young suckling AG129 mice showed neurological symptoms and even died with wild-type EV71-B4 infection because AG129 mice lack a functional interferon system and support both the spread from the primary infection site and the persistence of EV71 (30). As IFNs play an important role in the antiviral defense against EV71 infection, the \(LD_{50}\) differences between the parental RG/B4 and its high-fidelity variants described in AG129 mice might even be widened in an immunocompetent host, where fewer mutations in the population of high-fidelity variants might result in a lower likelihood of mutations capable of circumventing established innate and adaptive immune responses. Because EV71 fails to cause symptoms in AG129 mice more than 3 weeks old (30), it is hard to evaluate the efficacy of high-fidelity variants as live attenuated vaccines against EV71 in this mouse model. Actually, lacking cogent animal models for EV71 infection is hampering the research and development of EV71 vaccines although some new models seem to be promising (32, 49).

The conventional methods to develop attenuated viral vaccines are based on the identification of viruses with an overall lower fitness via passage in cells. However, the chance of the attenuated virus being outcompeted by fitter and more pathogenic revertants makes live attenuated vaccines less acceptable, especially for human usage. However, live attenuated EV71 vaccines should outweigh the use of inactivated EV71 vaccines for their lower cost and higher efficacy to elicit both cellular and humoral responses, and they would be more effective against EV71 outbreaks in developing countries with huge populations in the Asia-Pacific region, according to the experience of a polio eradication program (22, 23). Here, we showed that the attenuated high-fidelity EV71 variants with robust replication profiles (Fig. 6A) would contribute to the
development of both live attenuated and inactivated EV71 vaccines as high fidelity can reduce the mutation risk in the production of inactivated EV71 vaccines from wild-type strains (19–21).

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