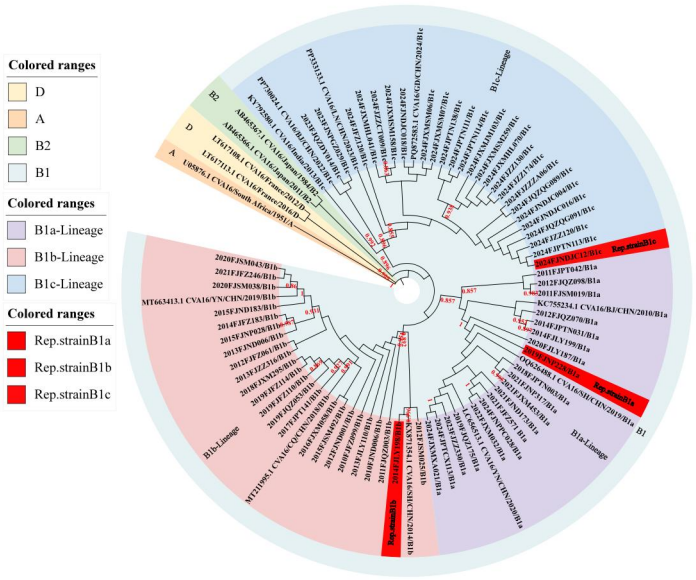
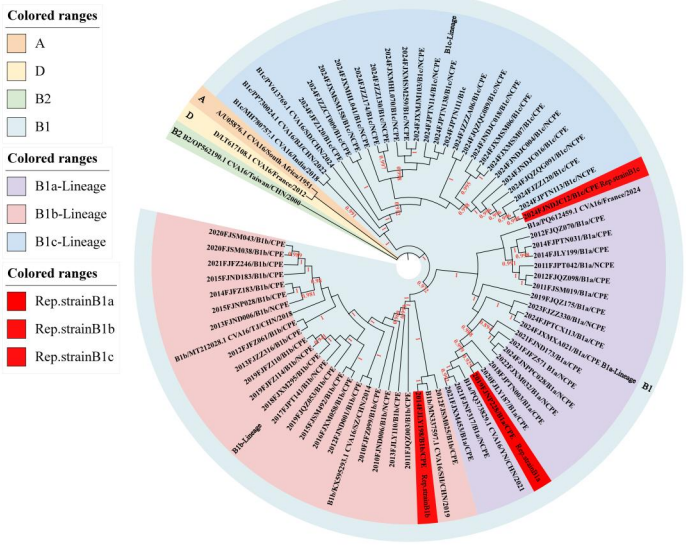
Supplementary Material

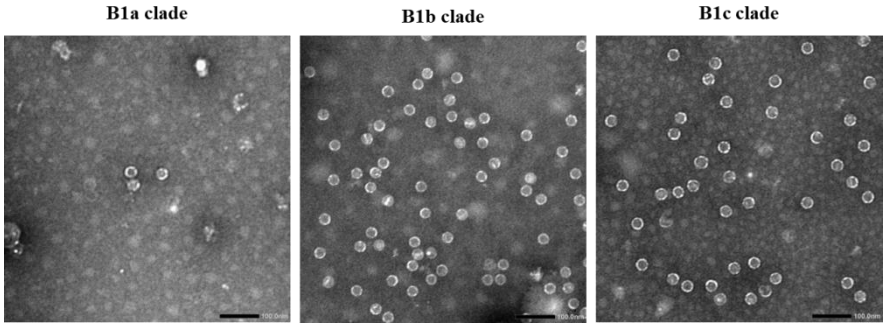


Supplementary Figure S1. Maximum Likelihood phylogenetic tree constructed based on the VP1 sequences of 67 CVA16 strains from Fujian Province (2010–2024) and 15 representative global CVA16 VP1 sequences. The outer circle represents different genotypes/subgenotypes, with red indicating the selected reference strains. CVA16, Coxsackievirus A16.

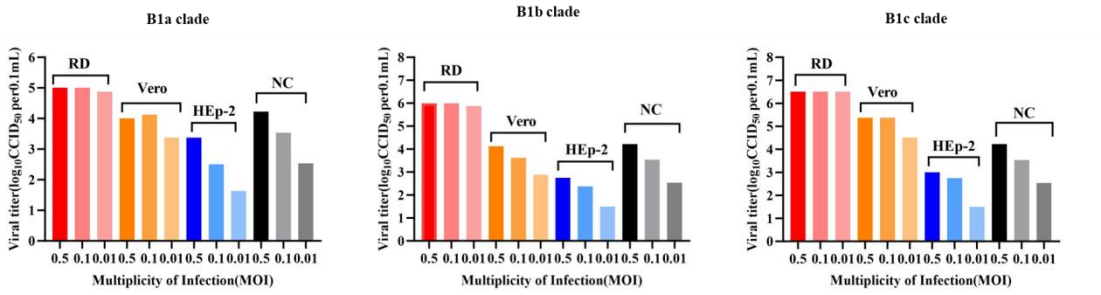


Supplementary Figure S2. Maximum Likelihood phylogenetic tree constructed based on the full-genome sequences of 65 CVA16 strains from Fujian Province (2010–2024) and 11 representative global CVA16 full-genome sequences. The outer circle represents different

genotypes/subgenotypes, with red indicating the selected reference strains. CVA16, Coxsackievirus A16.



Supplementary Figure S3. Purification and negative-stain electron microscopy analysis of B1a, B1b, and B1c clades.

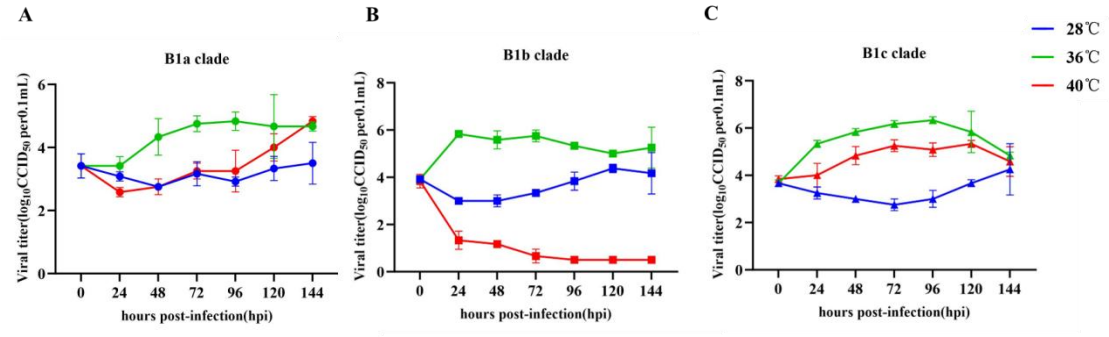


Supplementary Figure S4. Susceptibility of RD, Vero, and HEp-2 cells to infection by CVA16 B1a, B1b, and B1c clades at three different MOIs (Endpoint viral titers measured at 144 hpi). CVA16, Coxsackievirus A16.

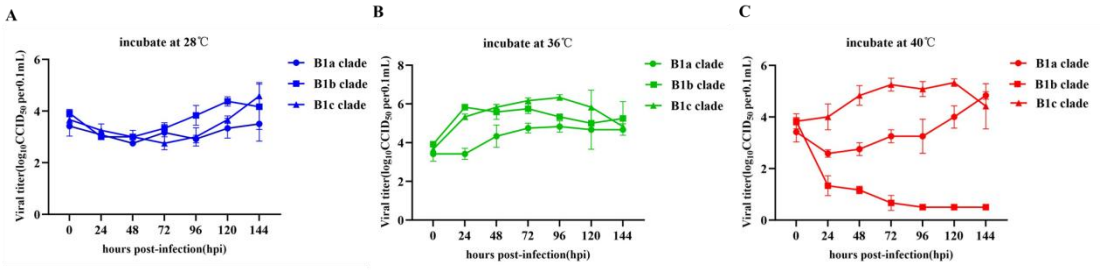
Supplementary Table S1. CPE induction in RD cells Infected with CVA16 subgenotype B1 at 0–144 hpi under different temperature culture conditions

	28°C	**36**°C			**40**°C		
Clade/h	**B1a/B1b/B1c**	**B1a**	**B1b**	**B1c**	**B1a**	**B1b**	**B1c**
0	−	−	−	−	−	−	−
24	−	−	+	−	−	−	−
48	−	+	++	+	−	−	−
72	−	++	+++	+++	−	−	+
96	−	+++	++++	++++	−	−	+++
120	−	++++	++++	++++	++	−	++++
144	−	++++	++++	++++	+++	−	++++

Note. "-" indicates no CPE; "+" indicates mild CPE (25%–50%); "++" indicates moderate CPE (50%–75%); "+++" indicates severe CPE (> 75%); "++++" indicates severe CPE (100%). CVA16, Coxsackievirus A16; CPE, cytopathic effect.



Supplementary Figure S5. Replication kinetics ($CCID_{50}$) of CVA16 B1a, B1b, and B1c clades from the same clade at different temperatures over 144 hours. (A) Representative strain of the B1a clade. (B) Representative strain of the B1b clade. (C) Representative strain of the B1c clade. Data points represent the mean viral titer ± standard deviation (SD) of three replicate samples. Statistical significance was determined by one-way *ANOVA* (ns, not significant, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). $CCID_{50}$, 50% cell culture infectious dose.



Supplementary Figure S6. *In vitro* replication kinetics of CVA16 B1a, B1b, and B1c clades cultured at different temperatures. Time course of viral titers for representative strains of the B1a, B1b, and B1c clades incubated at (A) 28°C, (B) 36°C, and (C) 40°C over a period of 0 to 144 hpi. Data are presented as the mean ± standard deviation of three biological replicates. Statistical significance was determined by one-way *ANOVA* (ns, not significant, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). CVA16, Coxsackievirus A16.

1. Materials and Methods

1.1 Data and Specimen Sources

The information of HFMD cases in Fujian Province was obtained from the Infectious Disease Surveillance Information Management System of the National Disease Surveillance Information Reporting System of China. Clinical specimens of HFMD and relevant epidemiological data were

provided by the HFMD Laboratory Surveillance System of Fujian Province. The reference sequence information of HFMD viruses was retrieved from the GenBank public database (https://www.ncbi.nlm.nih.gov/), which was used for subsequent sequence alignment and analysis.

1.2 Cell lines and Culture Passages

RD (human rhabdomyosarcoma) and HEp-2 (human laryngeal epidermoid carcinoma) cells were provided by the Polio Department of the National Institute for Viral Disease Control and Prevention, Chinese CDC. Vero (African green monkey kidney) cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) with the catalog number CCL-81. RD cells were used between passages 30–45, Vero cells between passages 25–40, and HEp-2 cells between passages 20–35. All cells were used within 15 passages after thawing.

1.3 Methods

1.3.1 Selection of Representative Strains of CVA16 with Three Evolutionary Clades

Full-length VP1 sequences and complete genomes of the three CVA16 clades obtained from Fujian Province (2010–2024) were aligned with the prototype strain G-10 and representative domestic and international strains retrieved from GenBank. Mean distances within and between groups for 1,000 bootstrap replicates were calculated using the p-distance model in MEGA X. The online tool iTOL website (https://itol.embl.de/) was used for post-annotation of the constructed evolutionary tree.

1.3.2 Virus Isolation and Cultivation

Clinical specimens of HFMD submitted by Centers for Disease Control and Prevention (CDCs) in various cities of Fujian Province were used for virus isolation and cultivation in RD cells. Virus isolation was performed accordance to the standard procedures in the Laboratory Manual for HFMD and two blind passages were conducted.

1.3.3 Plaque Purification and Titration of CVA16 Viruses

RD cell monolayers were infected with 200 µL of serially diluted virus (10^{-1}–10^{-5}), After adsorption at 36°C with 5% CO_2 for 2 h, the inoculum was removed. Each well was overlaid with 2 mL of 0.5% agarose-containing medium (a 1:1 mixture of 2 × MEM and 1% low-melting-point agarose). Plates were incubated at 36°C with 5% CO_2 for 3 days. Viral titers were determined according to the standard procedures in the Laboratory Manual for HFMD, and virus titers were calculated using

the Behrens-Kärber formula.

1.3.4 Purification and Transmission Electron Microscopy (TEM) Identification of CVA16 Viruses

CVA16 viruses were purified by referring to the protocol as follows. Briefly, RD cells were infected with clade-representative CVA16 strains at a multiplicity of infection (MOI) of 0.1. When more than 75% of cytopathic effect (CPE) was observed, the supernatant was collected for precipitation. The supernatant was treated with 50% polyethylene glycol (PEG) 8000 and 200 mmol/L NaCl at 4°C overnight. The precipitate was collected by centrifugation, resuspended in 0.15 mol/L phosphate-buffered saline (PBS) buffer, and stirred at 4°C overnight. After centrifugation, the supernatant was added to 20% sucrose buffer and ultracentrifuged at 27,000 rpm for 3 h. The pellet was resuspended in 0.15 mol/L PBS buffer. Purified virus suspensions were negatively stained with 2% phosphotungstic acid and observed under a TEM at 100 kV.

1.3.5 Sensitivity of CVA16 Viruses of Subgenotype B1 to RD, Vero, and HEp-2 Cells

The replication efficiency of three clade-representative CVA16 strains was assessed in RD, Vero, and HEp-2 cells. Cells were infected at MOIs of 0.5, 0.1, and 0.01. When > 75% CPE was observed, the culture was harvested; if no CPE appeared at 144 hpi, the supernatant was collected regardless. After three freeze-thaw cycles, viral titers were determined by the $CCID_{50}$ assay (as in section 1.3.3).

1.3.6 In Vitro Replication Kinetics and Temperature Sensitivity of CVA16 Viruses

RD cells were infected at an MOI of 0.1 and incubated at 28°C, 36°C, and 40°C with 5% CO_2, respectively. The experiment was performed in three independent biological replicates. Seven time points (0, 24, 48, 72, 96, 120, and 144 hpi) were selected. CPE was recorded, and virus titers in the supernatants (determined as in section 1.3.3) were used to generate replication curves.

1.3.7 Cytotoxicity of CVA16 Viruses to RD Cells

RD cells (3×10^4cells/well in a 96-well plate) were infected at an MOI of 0.1. At 6, 24, 48, 72, and 96 hpi, the medium was removed and replaced with fresh medium containing 10% CCK-8 reagent. After 1 h incubation at 36°C, absorbance at 450 nm was measured. Cell viability was calculated relative to mock-infected controls (set as 100%). Six replicates were used per condition.

1.3.8 Statistical Analysis

All analyses were performed using GraphPad Prism 10.1.2. Data are presented as mean ± standard error (SE). Multiple group comparisons were made by one-way or two-way analysis of

variance (*ANOVA*). *P*-value < 0.05 was considered statistically significant.