EMi   Taylor & Francis
Taylor & Francis Group

A screening study on the detection strain of Coxsackievirus A6: the key to evaluating neutralizing antibodies in vaccines

Fan Gao[a,b]‡, Pei Liu[c]‡, Yaqian Huo[b,d], Lianlian Bian[b], Xing Wu[b], Mingchen Liu[b], Qian Wang[b], Qian He[b], Fangyu Dong[e], Zejun Wang[f], Zhongping Xie[g], Zhongyang Zhang[h], Meirong Gu[i], Yingzhi Xu[i], Yajing Li[j], Rui Zhu[k], Tong Cheng [k], Tao Wang[a], Qunying Mao[b] and Zhenglun Liang[b]

[a]School of Life Sciences, Tianjin University, Tianjin, People's Republic of China; [b]Division of Hepatitis and Enterovirus Vaccines, National Institutes for Food and Drug Control, Beijing, People's Republic of China; [c]National Center for AIDS/STD Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China; [d]Department of Research & Development, Shanghai Institute of Biological Products Co., Ltd, Shanghai, People's Republic of China; [e]Department of Research & Development, Taibang Biologic Group, Beijing, People's Republic of China; [f]Department of R&D, Wuhan Institute of Biological Products Co., LTD, Wuhan, People's Republic of China; [g]Department of Production Management, Institute of Medical Biology, Chinese Academy of Medical Sciences, Kunming, People's Republic of China; [h]The Second Research Laboratory, National Vaccine and Serum Institute, Beijing, People's Republic of China; [i]R&D Center, Minhai Biotechnology Co., LTD, Beijing, People's Republic of China; [j]R&D Center, Sinovac Biotech Co., LTD, Beijing, People's Republic of China; [k]State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen, People's Republic of China

ABSTRACT
The increasing incidence of diseases caused by Coxsackievirus A6 (CV-A6) and the presence of various mutants in the population present significant public health challenges. Given the concurrent development of multiple vaccines in China, it is challenging to objectively and accurately evaluate the level of neutralizing antibody response to different vaccines. The choice of the detection strain is a crucial factor that influences the detection of neutralizing antibodies. In this study, the National Institutes for Food and Drug Control collected a prototype strain (Gdula), one subgenotype D1, as well as 13 CV-A6 candidate vaccine strains and candidate detection strains (subgenotype D3) from various institutions and manufacturers involved in research and development. We evaluated cross-neutralization activity using plasma from naturally infected adults ($n = 30$) and serum from rats immunized with the aforementioned CV-A6 strains. Although there were differences between the geometric mean titer (GMT) ranges of human plasma and murine sera, the overall trends were similar. A significant effect of each strain on the neutralizing antibody test (MAX/MIN 48.0 ~16410.3) was observed. Among all strains, neutralization of the S112 strain by 15 different sera resulted in higher neutralizing antibody titers ($GMT_{S112} = 132.0$) and more consistent responses across different genotypic immune sera (MAX/MIN = 48.0). Therefore, S112 may serve as a detection strain for NtAb testing in various vaccines, minimizing bias and making it suitable for evaluating the immunogenicity of the CV-A6 vaccine.

Introduction

Coxsackievirus A6 (CV-A6) is a small icosahedral RNA virus belonging to the human Enterovirus A species (EV-A) within the Picornaviridae family [1]. It is associated with a spectrum of human diseases, ranging from mild conditions, such as hand, foot, and mouth disease (HFMD) [2–8] and herpangina [9,10], to more severe manifestations, such as atypical HFMD [11–15], onychomadesis [12,15,16], and rare neurological complications [6,16–19]. These conditions can affect individuals of all ages, including children and adults [11,12,16,20,21]. Historically, HFMD outbreaks have predominantly been caused by Enterovirus 71 (EV-A71) and CV-A16 [22,23]. After 2010, CV-A6 has become the leading viral pathogen in numerous countries, causing HFMD outbreaks [2–41] (Figure 1). Han et al. [42] analyzed HFMD surveillance data in Jiangsu Province, China, from 2009 to 2020 and identified the emergence of CV-A6 as a pathogen causing severe cases in 2017. This proportion gradually increased, and by 2020, it accounted for approximately one-third of all pathogens causing severe HFMD, resulting in a large burden on individuals. Vaccines are a highly efficacious approach for mitigating disease severity and reducing mortality.

Continent	Country	Area	Year	Positive rate (%)	No. of EV positive	Ref.
ASIA	Thailand	/	2019-2022		368	24
	Japan	Fukuoka	2013-2017		202	25
	Japan	Osaka	2017		24	26
	India	/	2015-2017		107	27
	Malaysia	/	2018		50	5
	China	Taiwan	2010		275	28
	China	Beijing	2011-2020		2489	29
	China	Shanghai	2012-2016		800	30
	China	Guangdong province	2013		201	31
	China	Shenzhen	2013		1976	32
	China	Beijing	2013		130	33
	China	Tianjin	2013		440	34
	China	Changchun	2013		6234	35
	China	Shanghai	2014-2016		7010	36
	China	Wuhan	2015-2017		206	17
	China	Shanghai	2016-2017		1364	7
	China	Beijing	2016-2020		202	37
	China	Chengdu	2017-2022		913	38
AMERICA	Cuba	/	2011-2013		22	8
	Brazil	Northern	2018		35	19
	Brazil	Amazon region	2019		22	15
	Brazil	São Paulo	2021		139	3
EUROPE	Spain	/	2010-2012		62	39
	Spain	Barcelona	2017-2019		124	40
	Hungary	/	2010-2018		284	41
	France	/	2021		190	2
	Italy	/	2012-2014		11	14
	Denmark	/	2014-2016		23	12
	United Kingdom	/	2010-2014		55	11
OCEANIA	New Zealand	Auckland	2013		22	16

■ EV-A71 positive ■ CV-A16 positive ■ CV-A6 positive ■ CV-A10 positive ■ Others
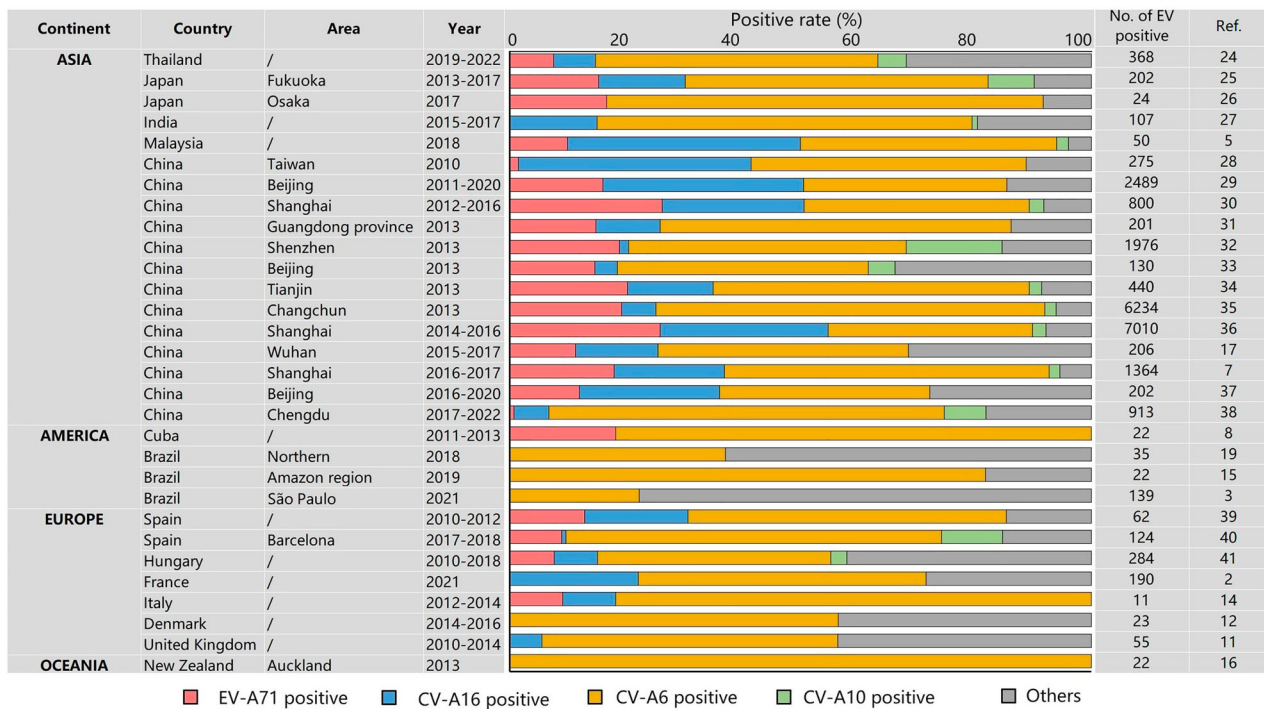
Figure 1. Epidemiology of HFMD since 2010.

Since December 2015, HFMD vaccines, including inactivated whole- virus EV-A71 vaccines manufactured by three Chinese manufacturers, have been approved for marketing to control severe and lethal HFMD [43,44]. However, developing of a monovalent EV-A71 vaccine is only an initial step toward comprehensive HFMD control [44]. Owing to the limited cross-protection between different EV-A serotypes [44], relying solely on a monovalent EV71 vaccine cannot adequately control CV-A6-induced HFMD. Currently, global institutions are endeavoring to develop CV-A6 vaccines [45–50]. Zhou et al. [45] produced a monovalent CV-A6 virus-like particle (VLP) vaccine using *Pichia pastoris* yeast transformed with a vector encoding both the P1 and 3CD proteins of CV-A6. Immunization with CV-A6 VLPs elicited CV-A6-specific serum antibodies in mice. Passive transfer of anti-VLP sera protected recipient mice against lethal CA6 challenge. Additionally, studies have been conducted to develop multivalent HFMD vaccines containing CV-A6 by mixing different EV monovalent vaccines, including bivalent [46], trivalent [47–49], and quadrivalent vaccines [50] (Table 1).

The correlation between neutralizing antibodies (NtAbs) and protection has been consistently demonstrated in numerous studies on enteroviruses [43,44,51–55], emphasizing the pivotal role of accurate detection of neutralizing antibodies in vaccine evaluation. Therefore, objective and accurate evaluation of the NtAb response level to different vaccines is challenging, given the concurrent development of multiple vaccines in China and worldwide [45–50], each utilizing distinct vaccine strains. The choice of the detection strain is a crucial factor that influences the detection of NtAbs. Research on EV-A71 [51] and CV-A10 [56] has demonstrated that differences in test results can be as high as 384-fold and 4096-fold, respectively, when using distinct detection strains. However, this topic has not yet received sufficient attention. In this study, the National Institutes for Food and Drug Control (NIFDC), a national laboratory for vaccine quality control in China, collected a prototype strain (Gdula), one subgenotype D1, as well as 13 CV-A6 candidate vaccine strains and candidate detection strains (subgenotype D3) from various institutions and manufacturers involved in research and development (R&D). We identified a detection strain that exhibited higher NtAb titers and more consistent responses across different genotypic immune serum samples. It has the potential to serve as a detection strain for NtAb testing in various vaccines, minimizing bias and making it suitable for evaluating the immunogenicity of the CV-A6 vaccine.

Moreover, an effective vaccine should be able to induce a broad spectrum of neutralizing activities against various strains [51]. However, obtaining broad-spectrum efficacy data is challenging because single manufacturers have a limited number of strains. This study aimed to compare the cross-neutralization activities of antibodies induced by 15 CV-A6 strains in order to understand the differences among various viral strains and provide valuable reference data for manufacturers regarding the broad-spectrum efficacy of these strains.

Table 1. Summary of the research and development of the CV-A6 vaccine

Vaccine	Year	Component	Type	CV-A6 strain	CV-A6 GenBank ID	Cell	Stage	Institution	Country /Region	Ref.
Monovalent	2016	CV-A6	VLP	SZc173/13	KF682362.1	*Pichia pastoris*	Pre-clinical	Institut Pasteur of Shanghai Chinese Academy of Sciences	Mainland China	[45]
Bivalent	2018	CV-A6, CV-A10	INA	/	/	RD	Pre-clinical	Taishan Medical University	Mainland China	[46]
Trivalent	2015	EV-A71, CV-A16, CA6	INA	Gdula	AY421764.1	RD/Vero	Pre-clinical	University of Wisconsin-Madison	United States	[47]
	2016	EV-A71, CV-A16, CA6	INA	M0746	/	RD	Pre-clinical	National Health Research Institutes	Taiwan, China	[48]
	2018	CV-A6, CV-A10, CV-A16	INA	/	/	RD/Vero	Pre-clinical	National Institute of Health	South Korea	[49]
Quadrivalent	2018	EV-A71, CV-A16, CV-A6, CV-A10	VLP	SZc173/13	KF682362.1	Sf9	Pre-clinical	Institut Pasteur of Shanghai Chinese Academy of Sciences	Mainland China	[50]
	/	EV-A71, CV-A16, CV-A6, CV-A10	INA	/	/	/	Pre-clinical	Sinovac Biotech Co., Ltd.	Mainland China	/
	/	EV-A71, CV-A16, CV-A6, CV-A10	INA	/	/	/	Pre-clinical	Wuhan Institute of Biological Products Co., Ltd.	Mainland China	/
	/	EV-A71, CV-A16, CV-A6, CV-A10	INA	/	/	/	Pre-clinical	Minhai Biotechnology Co., Ltd.	Mainland China	/
	/	EV-A71, CV-A16, CV-A6, CV-A10	INA	/	/	/	Pre-clinical	Wantai BioPharm/Xiamen University	Mainland China	/
	/	EV-A71, CV-A16, CV-A6, CV-A10	INA	/	/	/	Pre-clinical	National Vaccine and Serum Institute	Mainland China	/
	/	EV-A71, CV-A16, CV-A6, CV-A10	VLP	/	/	/	Pre-clinical	Huasong (Shanghai) Biomedical Technology Co., Ltd.	Mainland China	/

INA: Inactivated vaccine. VLP: Virus like particles vaccine. /: Not available.

Materials and methods

Viruses and cells

A total of 15 CV-A6 viruses were collected. Gdula was purchased from the American Type Culture Collection (ATCC). XM and S101 were propagated from viral samples preserved at the NIFDC. Others were kindly provided by the Institute of Medical Biology, Chinese Academy of Medical Sciences, National Vaccine and Serum Institute, Wuhan Institute of Biological Products Co., Ltd., Minhai Biotechnology Co., Ltd., and Sinovac Biotech Co., Ltd.. All viruses were propagated in rhabdomyosarcoma (RD) cells using MEM solution (Gibco, USA) containing 2% (v/v) fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), and 100 IU/ml penicillin and streptomycin (Gibco). The 50% cell culture infective dose ($CCID_{50}$) was determined using RD cells, and the values were calculated using the Karber formula [57]. Details of the viral strains used in this study are presented in Table 2.

The CV-A6 sequences in the VP1 region were downloaded from the National Center for Biotechnology Information (NCBI). Sequences containing clear information, such as isolation time and location, were selected for further analysis, and the VP1 region sequences were subjected to cluster analysis using a clustering similarity threshold of 0.97. During the same isolation year, 1–5 sequences were selected as representative sequences from the same sampling country, region, or province, and 364 CV-A6 VP1 sequences containing accurate sampling information were obtained. A phylogenetic tree was constructed using the VP1 sequences of 15 CV-A6 candidate vaccine strains and the candidate detection strains were collected, and 364 viral sequences were curated. MEGA7 software was used for genotyping. A distance of less than 0.15 between groups was considered the same genotype, and a distance of less than 0.08 was considered the same subgenotype. Representative sequences of each genotype and 15 CV-A6 sequences were selected, and the Maximum Likelihood (ML) method was used to construct a phylogenetic tree. First, the optimal nucleic acid replacement model was calculated as SYM + R4 using IqTree 1.6.2. An ML tree was constructed using a bootstrap value of 1000. We tagged the ML tree using Adobe Illustrator CC 2019 23.0. Homology was analyzed using an online tool (https://www.ebi.ac.uk/Tools/msa/Clustalo/).

Animal

Wistar rats and one-day-old suckling mice (BALB/c) were purchased from the Laboratory Animal Resources Institute, NIFDC. All animal experiments were approved by the NIFDC Ethics Committee before the commencement of the study [NIFDC-2020(B)018] and [NIFDC-2023(B)044].

Serum samples

Plasma

Thirty plasma samples collected from naturally infected adults between January and May 2019 in Cao County, Shandong Province, China, were gifted by the Taibang Biologic Group. Written informed consent was obtained from the guardians of all donors.

Serum samples from rats inoculated with different CV-A6 viruses

Anti-Gdula, anti-XM, anti-S101, anti-S102, anti-S104 to S110, anti-S112, and anti-S114 sera were collected from female Wistar rats (specific pathogen free) aged 6–8 weeks and immunized with the respective CV-A6 strains (three rats/strain) via the intraperitoneal (i.p.) route at an approximate dose of $10^{7.5}$ $CCID_{50}$. Immunizations were performed at weeks 0, 2, and 4, and blood samples were collected at week 5 and centrifuged at $4,000 \times g$ at 2–8 °C for 30 min to obtain sera. The sera were stored at −20 °C. Anti-S113 and anti-S103 serum samples were provided by the Wuhan Institute of Biological Products Co., Ltd.

Measurement of CV-A6 cross-NtAbs

CV-A6 NtAb levels in the serum samples (or plasma samples) were measured using the cytopathogenic effect (CPE) method. Two-fold serial dilutions of serum (started at l:8) were incubated with an equal volume of virus culture (50 μL) containing 100 $CCID_{50}$/well of CV-A6 strains at 37 °C for 2 h. RD cell suspension (final concentration: 2.0×10^5 cells/mL) was then added, and the mixture was incubated in a CO_2 incubator at 35 °C for seven days before the CPE was observed using microscopy. Neutralizing titers were defined as the highest dilutions required to achieve 50% CPE inhibition.

Cross-protection

BALB/c suckling mice aged 1 d (5–8/group) were challenged by lethal doses (30 LD_{50}) of 20 μL via i.p., followed by injection with the diluted protective serum via i.p. within 1 h. After 21 days of continuous observation, deaths were recorded to evaluate the passive protective effects of the serum in vivo.

Statistical analysis

Microsoft Excel 2020, SPSS Statistics 21, and GraphPad Prism 8.0 were used for statistical processing and data analyses. A CV-A6 NtAb titer <8 was considered negative, whereas a CV-A6 NtAb titer ≥8

was considered positive. The NtAb titer <8 was set to 4 during the calculation. NtAb titers were log-transformed to calculate the geometric mean titer (GMT). The value of MAX/MIN refers to the ratio of the maximum and minimum values of NtAbs against each strain detected in the serum or plasma. The NtAb of each antiserum against counterpart strain was unified by multiplying a coefficient and shown as 100 eventually, and NtAbs against other strains were calculated using the same calculation. The LD_{50} was calculated using the Karber formula [57].

Results

Genotyping

The prototype A/Gdula was purchased from ATCC, and 14 CV-A6 strains were obtained from institutions and manufacturers involved in R&D in China. These strains were isolated from different regions of China between 2016 and 2019. In total, 364 CV-A6 VP1 sequences were downloaded from the NCBI database. A phylogenetic tree was constructed using the VP1 sequences of the 15 CV-A6 strains collected in this study and 364 viral strains obtained from the NCBI database (Figure 2). The results showed that 13 strains isolated from mainland China from 2016 to 2019 belonged to the dominant D3 subgenotype branch, and the XM strain isolated from Chinese Taiwan belonged to the dominant D1 subgenotype branch. In addition, the D3 subgenotype exhibited a range of 94.21%–99.78% sequence identity among its members while showing the identity of 82.19%–83.61% with genotype A and 88.52%–90.05% with the D1 subgenotype. The homology between A and D1 was 82.40%.

Screening detection strain

Cross-neutralization activities of different CV-A6 strains on the plasma of naturally infected humans

Cross-neutralizing antibody activity against CV-A6 strains was assessed in 30 plasma samples collected from naturally infected humans living in Cao County, Shandong Province, mainland China, between January and May 2019. Fifteen CV-A6 strains were used to detect the NtAbs. As shown in Figure 3(A), the neutralization activity of plasma against all strains varied, with GMT of neutralizing antibodies against all strains ranging from 18.1–112.2. The variation ranged from 1.0- to 6.2-fold ($GMT_{S106}/GMT_{S104} = 1.0$, $GMT_{S110}/GMT_{S107} = 6.2$); the variation between subgenotype D3 and subgenotype D1 ranged from 0.3- to 1.6-fold ($GMT_{S107}/GMT_{XM} = 0.3$, $GMT_{S110}/GMT_{XM} = 1.6$); the variation between subgenotype D3 and genotype A ranged from 0.5- to 3.4-fold ($GMT_{S107}/GMT_{Gdula} = 0.5$, $GMT_{S110}/GMT_{Gdula} = 3.4$); and the variation

between subgenotype D1 and genotype A was 2.1-fold (GMT_{XM}/GMT_{Gdula}). The order of GMTs against the different strains, from low to high, was as follows: S107 < S108 < S104 < S106 < Gdula < S109 < S113 < S105 < S112 < S102 < S114 < S101< XM < S103 < S110. The highest NtAb GMT was observed for S110 ($GMT_{S110} = 112.2$), and the lowest GMT was observed for S107 ($GMT_{S107} = 18.1$), indicating that S107 was more difficult to be neutralized than the other strains. Among the remaining 14 strains, there was a maximum fold variation of 3.7 (GMT_{S110}/GMT_{S104}), suggesting a certain degree of similarity between these strains.

Cross-neutralization activities of different CV-A6 strains in murine sera

Serum samples were collected after immunizing Wistar rats with the 15 CV-A6 strains separately. Each serum sample was tested to determine its cross-neutralization ability against various CV-A6 strains. Although there were differences between the GMT ranges of the human plasma and murine sera, the overall trends were similar. As shown in Figure 3(B), the GMTs of 15 sera detected with 15 strains ranged from 63.8–886.8, with a 1.5- to 13.9-fold variation ($GMT_{S109}/GMT_{S107} = 1.5$, $GMT_{S102}/GMT_{S107} = 13.9$). The variation between subgenotypes D3 and D1 ranged from 0.5- to 6.7-fold ($GMT_{S107}/GMT_{XM} = 0.5$, $GMT_{S102}/GMT_{XM} = 6.7$); the variation between subgenotype D3 and genotype A ranged from 0.7–9.4 ($GMT_{S107}/GMT_{Gdula} = 0.7$, $GMT_{S102}/GMT_{Gdula} = 9.4$); and the variation between subgenotype D1 and genotype A was 1.4-fold (GMT_{XM}/GMT_{Gdula}). The order of GMTs against the different strains, from low to high, was as follows: S107 <Gdula < S109 < S108 < XM < S104 < S105 <S113 < S110 < S101 < S114 < S106 < S112 <S103 < S102. Among the D3 strains, S107 showed the lowest propensity to be neutralized compared with the other strains of subgenotype D3. Further investigation is warranted to elucidate the underlying mechanisms.

To mitigate the error originating from the different titers of each serum, the NtAb titers of each antiserum against its counterpart strain were standardized by converting them proportionally to 100 while appraising the impact of each strain on NtAb detection. As shown in Table 3, GMTs of the detection strains ranged from 11.7–176.9, and the fold was 15.1 over the GMT of S107, which had the lowest value ($GMT_{S107} = 11.7$) among all strains tested followed by S109 ($GMT_{S109} = 17.6$) and Gdula ($GMT_{Gdula} = 17.8$), indicating that genotype A and subgenotype D3/S109 were also hard to be neutralized. Conversely, S102, S112, and S106 exhibited the highest GMT values (176.9, 132.0, and 111.4, respectively), indicating their susceptibility to neutralization. The MAX/MIN values of each strain against all sera were calculated to screen for homogeneously reactive strains with any serum
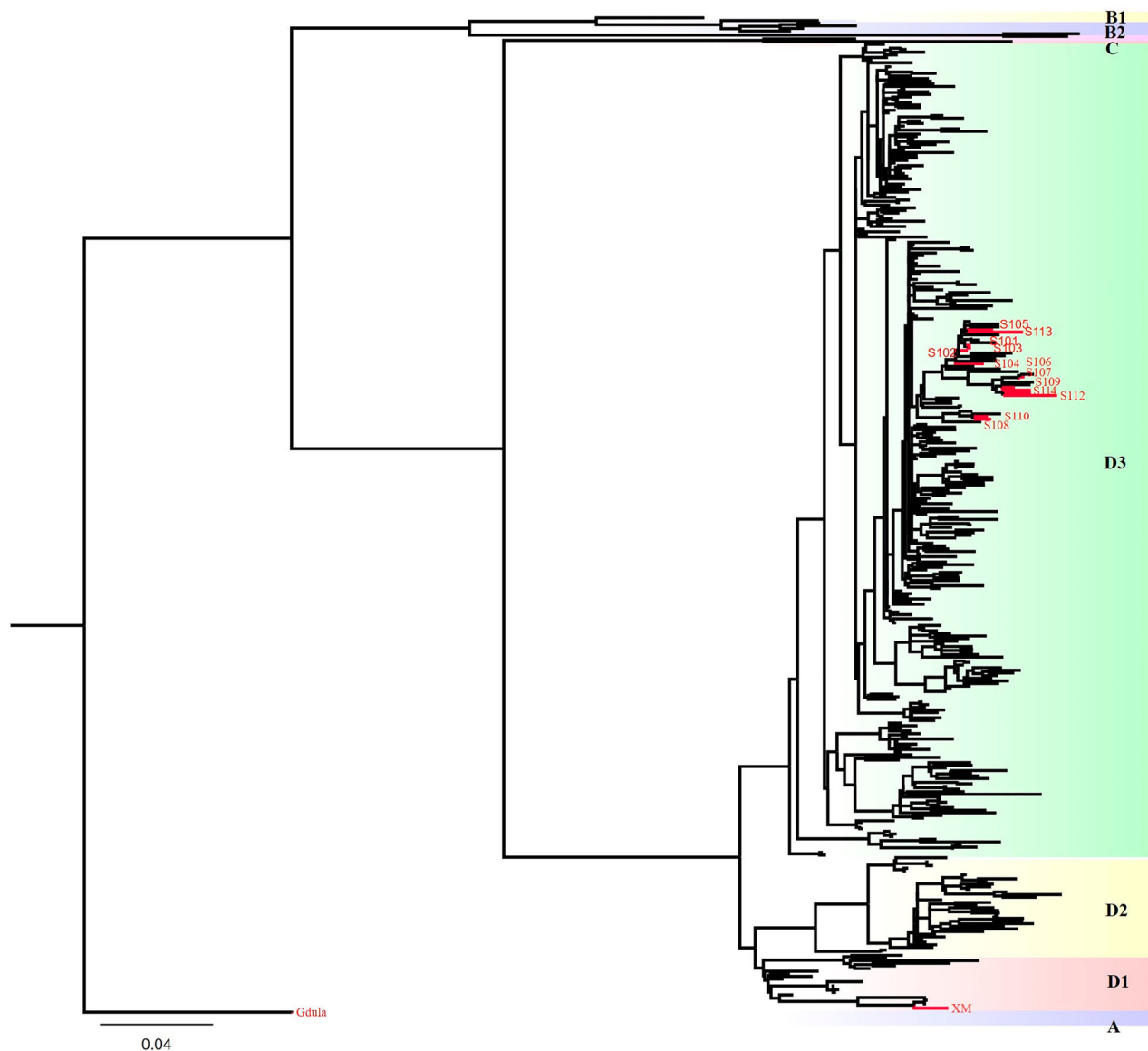
Figure 2. The genotyping of the 15 CV-A6 vaccine and detection candidate strains used in this study. The ML tree analysis revealed that the prototype Gdula strain belongs to genotype A, while the 13 CV-A6 strains belong to subgenotype D3, and the XM strain belongs to subgenotype D1. The names of these strains are indicated in the figure.

(Table 3). Screening of the detection strain was conducted to evaluate its susceptibility to neutralization by different sera, focusing on minimizing the MAX/MIN value, which indicated equal and desirable neutralization potential. The results demonstrated that the MAX/MIN of S103 was the highest (16410.3), indicating that the relative discrepancies in the neutralization of S103 by different sera were the most evident. Conversely, that of strain S112 was minimal (48.0). This suggests that S112 exhibits a more consistent response across all sera and may serve as a detection strain for different vaccines.

The difference in broad-spectrum efficacy among CV-A6 strains

As shown in Figure 3, the NtAb titer of the serum immunized by Gdula (anti-Gdula) and XM (anti-

XM) was positive for all 15 CV-A6 strains (NtAb titer ≥ 8), suggesting that the immune sera of A and D1 had cross-neutralized with all subgenotype D3 strains. The broad-spectrum neutralization activity of anti-S106 was found to be limited as it could not neutralize three out of the 15 CV-A6 strains tested (NtAb titer < 8); the NtAb titer of anti-S110 against all strains was positive, and the GMT was the highest (3153.3). Cross-neutralizing homogeneity was assessed by calculating the MAX/MIN value of each serum sample for all strains (Figure 4). A smaller MAX/MIN value indicated better cross-neutralizing ability of the serum derived from mice immunized with this strain. The MAX/MIN values of all the strains ranged from 8.0–512.0, indicating differences in the cross-neutralizing homogeneity of each serum. The sequence of MAX/MIN values from lowest to highest was as follows: anti-S113 < anti-S110 = anti-
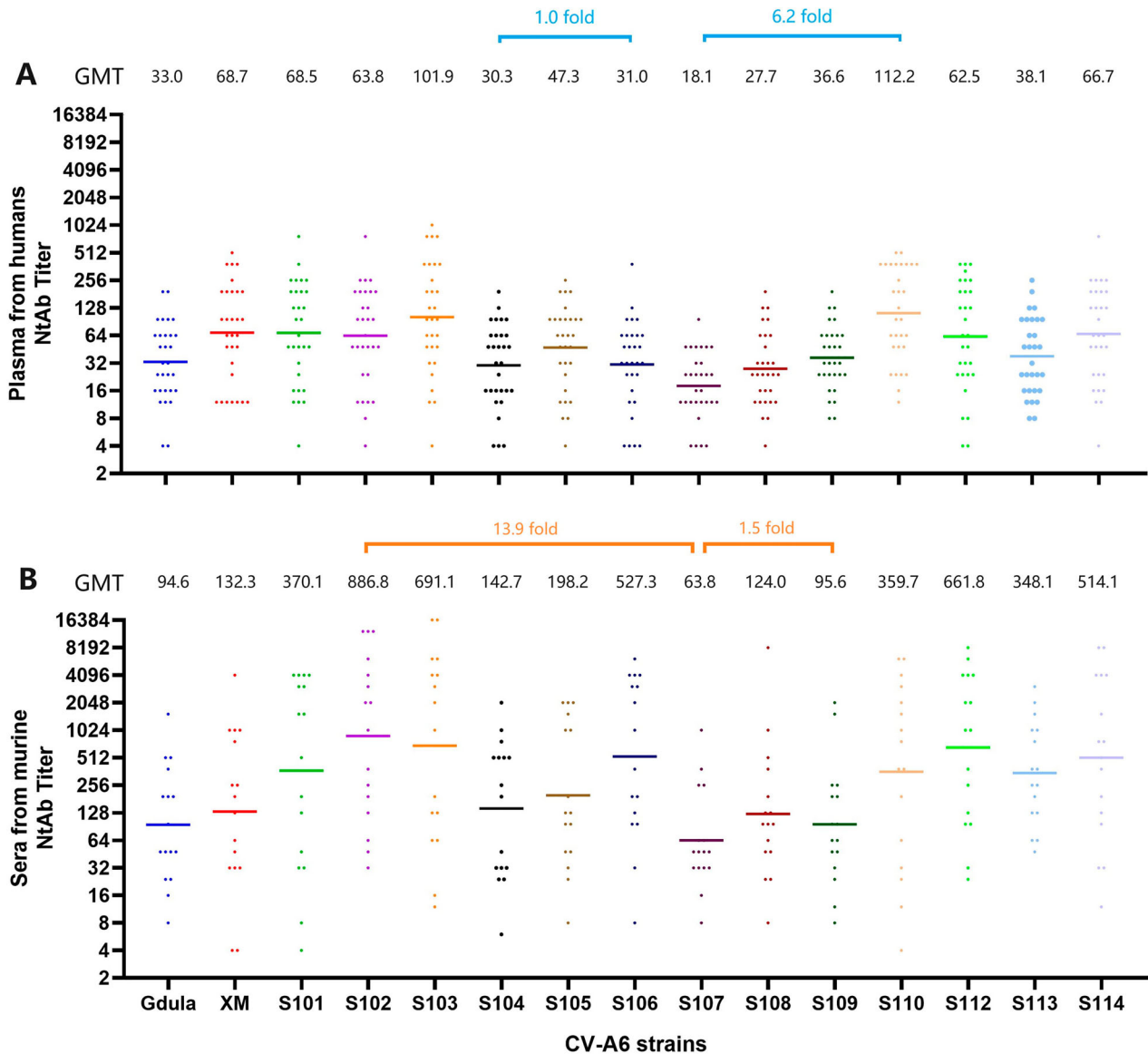
Figure 3. Cross-neutralizing capacity of the plasma from humans naturally infected with CV-A6 and the sera from murine immunized with CV-A6. A: The GMTs of 30 human plasma against various strains of CV-A6 ranged from 18.1 to112.2. The difference among the GMTs for genotype A, subgenotype D1, and C3 strains ranged from 1.0–6.2-folds (shown with a blue bar). B: The GMTs of 15 murine sera against various strains ranged from 63.8–886.8, of which the discrepancy among all strains was 1.5–13.9-fold (shown with an orange bar).

S106 < anti-S103 < anti-Gdula = anti-S109 < anti-S104 < anti-S114 < anti-S101 < anti-S112 = anti-S107 = anti-S108 < anti-S111 = anti-S102 < anti-XM = anti-S105. The MAX/MIN values of anti-XM and anti-S105 were the highest (512.0), suggesting that the cross-neutralization ability of these two sera varied against different strains. The MAX/MIN values of anti-S113, anti-S110, and anti-S106 were the lowest (8.0, 16.0, and 16.0, respectively), indicating that S113, S110, and S106 may have high neutralizing activity, broad-spectrum activity, and cross-neutralizing homogeneity.

To verify the *in vivo* cross-neutralization ability of serum immunized with S110, the anti-S110 serum was diluted 48-fold. One-day-old BALB/c mice were intraperitoneally challenged with lethal doses (30 LD_{50}) of Gdula, S101, S102, S110, or S112. A diluted anti-S110 serum was injected intraperitoneally within

1 h of the challenge. The results presented in Table 4 and Figure 5 demonstrate that a 48-fold dilution of anti-S110 exhibited a protective effect against lethal doses of all five CV-A6 strains (survival rates ranging from 67% to 100%), indicating its broad-spectrum cross-protective efficacy *in vivo*, which is consistent with the findings observed *in vitro*.

Discussion

All the CV-A6 strains were divided into several subgenotypes. However, no uniform standards have yet been established for this classification. Identifying of distinct CV-A6 genotypes, categorized as A-D [58] or A-F [59], relies on analyzing the VP1 sequence, with a primary sequence difference exceeding 15% serving as an indicator. Genotype B was further classified

Table 2. List of CV-A6 strains.

Name	Genotype	Year	Location[a]	Virus titer ($lgCCID_{50}$/mL)	LD_{50} ($lgCCID_{50}$/mL)
Gdula	A	1949	USA	7.62	3.62
XM	D1	2007	Taiwan province	8.17	N/A
S101	D3	2016	Hubei province	8.31	1.78
S102	D3	2016	Guangdong province	9.38	3.21
S103	D3	2016	Hubei province	8.44	N/A
S104	D3	2017	Beijing	8.38	N/A
S105	D3	2017	Beijing	8.44	N/A
S106	D3	2018	Beijing	8.63	N/A
S107	D3	2018	Beijing	8.31	N/A
S108	D3	2018	Beijing	8.31	N/A
S109	D3	2018	Beijing	9.44	N/A
S110	D3	2018	Guangdong province	8.94	2.19
S112	D3	2018	Hubei province	8.88	4.23
S113	D3	2018	Hubei province	7.85	N/A
S114	D3	2019	Guangdong province	8.50	N/A

[a]Gdula was isolated from the USA, whereas the others were isolated from China. LD_{50}: median lethal dose. N/A: not applicable.

as subgenotype B1 and B2, whereas genotype D was subdivided into subgenotypes D1–D3 [58]. The Gdula strain (GenBank ID: AY421764), identified as genotype A, was the first isolated CV-A6 strain in the USA in 1949 [58, 60]. However, it is not prevalent in the general population. The VP1 sequences of CV-A6 strains from multiple countries after 2008 revealed that, except for the Indian strain N-313 (subgenotype C2), all other international CV-A6 strains belonged to genotype D [58]. The outbreaks of CV-A6 observed in Europe from 2008 to 2010 can be attributed to two major subgenotypes (D1 and D3), with subgenotype D3 emerging as the predominant cause of most global outbreaks after 2010 [58]. In recent years, subgenotype D3 has caused HFMD outbreaks in Thailand [24], India [27], Brazil [3,19], and Spain [39,40]. In China, research [61] indicates that most CVA6 strains belong to the D genotype (95.6%), which mainly circulates in the eastern, northern, and southern regions of China. Subgenotype D3 first circulated in 2008 and has become the predominant subgenotype since 2009, reaching a peak in 2013, whereas D2 has been mostly undetectable in recent years [61]. Therefore, subgenotype D3 viruses are likely to exhibit enhanced transmissibility, infectivity, and virulence, potentially serving as the primary driver of sustained global circulation of CV-A6 [58]. To effectively implement outbreak prevention and control, various vaccine R&D institutions and manufacturers have selected subgenotype D3 (Table 2, Figure 2) as a candidate vaccine strain isolated from HFMD cases in three regions of mainland China (Guangdong Province, Hubei Province, and Beijing) from 2016 to 2019. Our results showed that the homology in the VP1 sequence of

Table 3. Comparison of serum cross-neutralization capacities after setting the value of serum neutralization against the corresponding virus strain to 100.

Murine Sera	CV-A6 strains														
	Gdula	S101	XM	S106	S107	S110	S112	S114	S102	S104	S105	S108	S109	S113	S103
anti-Gdula	**100.0**	33.3	33.3	200.0	33.3	25.0	400.0	100.0	266.7	33.3	50.0	50.0	33.3	133.3	12.5
anti-S101	9.4	**100.0**	18.8	25.0	1.2	100.0	100.0	100.0	50.0	12.5	50.0	3.1	4.7	25.0	1.6
anti-XM	75.0	75.0	**100.0**	150.0	75.0	300.0	150.0	300.0	200.0	50.0	75.0	75.0	150.0	200.0	25600.0
anti-S106	50.0	12.5	12.5	**100.0**	50.0	12.5	100.0	37.5	200.0	18.8	25.0	25.0	25.0	200.0	50.0
anti-S107	75.0	100.0	100.0	400.0	**100.0**	100.0	400.0	100.0	600.0	75.0	100.0	75.0	75.0	200.0	9600.0
anti-S110	25.0	50.0	66.7	33.3	16.7	**100.0**	33.3	133.3	66.7	16.7	33.3	133.3	33.3	50.0	266.7
anti-S112	4.7	75.0	6.3	75.0	1.2	50.0	**100.0**	18.8	150.0	6.3	3.1	2.3	1.6	18.8	4.7
anti-S114	6.3	50.0	12.5	50.0	3.1	75.0	100.0	**100.0**	150.0	25.0	18.8	12.5	18.8	12.5	75.0
anti-S102	1.6	12.5	2.1	50.0	0.4	6.3	33.3	12.5	**100.0**	1.6	1.6	0.8	0.8	3.1	33.3
anti-S104	6.3	16.7	25.0	25.0	8.3	50.0	33.3	66.7	50.0	**100.0**	16.7	25.0	25.0	33.3	3.1
anti-S105	1.2	200.0	6.3	200.0	3.1	50.0	300.0	200.0	600.0	25.0	**100.0**	18.8	12.5	100.0	266.7
anti-S108	400.0	3200.0	800.0	3200.0	200.0	2400.0	800.0	3200.0	2400.0	400.0	800.0	**100.0**	37.5	150.0	3.1
anti-S109	75.0	800.0	50.0	600.0	50.0	600.0	1600.0	600.0	1600.0	75.0	150.0	150.0	**100.0**	600.0	100.0
anti-S113	18.8	75.0	18.8	37.5	12.5	25.0	37.5	50.0	18.8	12.5	37.5	25.0	18.8	**100.0**	100.0
anti-S103	4.7	37.5	25.0	75.0	9.4	37.5	50.0	18.8	50.0	12.5	25.0	12.5	6.3	37.5	**100.0**
GMT	17.8	76.4	26.7	111.4	11.7	72.1	132.0	98.9	176.9	25.7	36.5	22.1	17.6	63.5	107.6
MAX/MIN	341.9	256.0	384.6	128.0	512.8	384.0	48.0	256.0	128.0	256.4	512.8	192.3	192.3	191.7	16410.3

Figure 4. Cross-neutralization ability of sera from murine inoculated with CV-A6 strains. Fifteen sera were collected after inoculating murine with 15 CV-A6 strains separately. CV-A6 NtAb titer < 8 (Log2 Scale < 3) was considered negative, while CV-A6 NtAb titer ≥ 8 (Log2 Scale ≥ 3) was considered positive.

subgenotype D3 ranged from 94.21% to 99.78%. The homology between subgenotypes D3 and D1 isolated from Chinese Taiwan in 2007 was 88.52%–90.05%, and 82.19%–83.61% with prototype A/Gdula, suggesting that they are largely different from genotype A.

Because of differences in the use of detection strains, comparing the efficacy of CV-A6 vaccines produced by manufacturers in China and several Asia-Pacific countries and regions is challenging. Previous studies have demonstrated that the choice of the detection strain used in the neutralization assay can significantly affect the NtAb titer [51,56]. Although selecting an appropriate detection strain

for estimating NtAb titers is crucial in vaccine R&D, it has not received adequate attention. To compare the differences in the test results caused by the different CV-A6 strains, we set the serum NtAb titer against the corresponding viral strain to 100. This approach helps minimize errors resulting from fluctuations in serum titers [51,56]. The MAX/MIN values of all strains against the sera were analyzed, assuming that a lower value indicated better homogeneity of detection. Our results showed that S112 exhibited the highest GMT (132.0) and lowest MAX/MIN value (48.0). Therefore, the S112 strain is more suitable as a detection strain for CV-A6 neutralization antibodies because it minimizes the bias introduced by other detection strains. This strain plays a crucial role in vaccine evaluation and provides a solid foundation for comparing various CV-A6 vaccines. Additionally, S112 exhibited the highest LD_{50} (4.23 $lgCCID_{50}$/mL) in mice, making it a potential challenge strain for future animal protection models *in vivo*, which may be used to evaluate *in vivo* efficacy and correlate with *in vitro* neutralization potency.

Table 4. Cross-protection of anti-S110 against the lethal challenge of five CV-A6 strains.

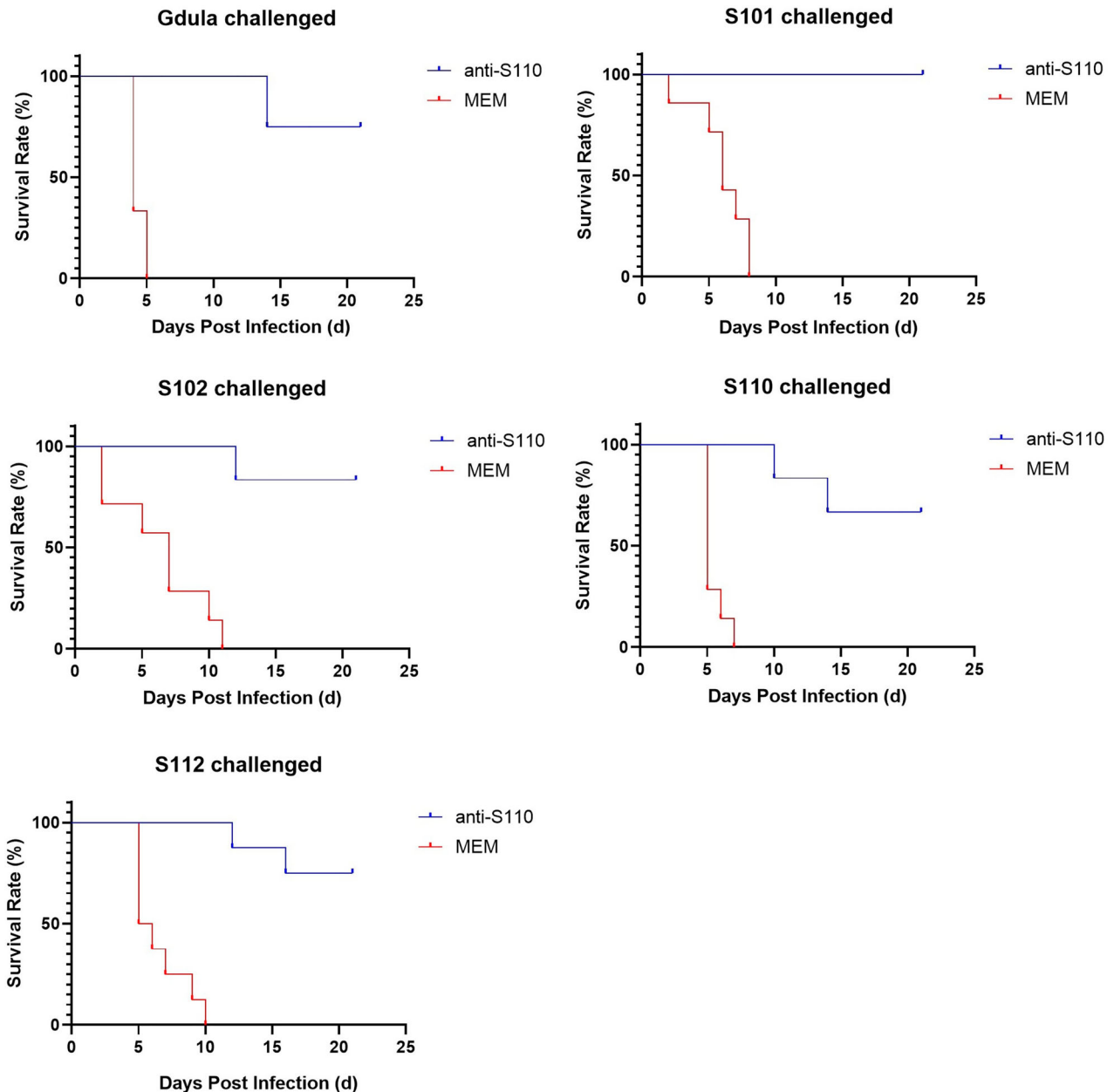| Item	CV-A6 strains				
	Gdula	S101	S110	S112	S102
NtAb titer *in vitro*	1536	3072	6144	2048	4096
Survival rate *in vivo* (%)	75	100	67	75	83

Figure 5. Cross protective effect of anti-S110 against lethal challenge of 5 CV-A6 strains. Anti-S110 (48-fold dilution) showed a good protective effect against 5 CV-A6 strains, and it ascended from 67% to 100% in the order S110, Gdula, S112, S102, and S101.

A significant effect of each strain on the NtAb test ($MAX/MIN_{S112} = 48.0$, $MAX/MIN_{S103} = 16410.3$) was observed. The large difference in MAX/MIN values suggests that there may be differences in the neutralization epitopes of these variants. However, studies of the neutralizing epitope of CV-A6 are limited. The information provided in Supplementary Table 1 reveals the identification of two CV-A6-specific linear B-cell epitopes, which were mapped to the GH loop (206–220 aa) and the C-terminal region (291–305 aa) of VP [62]. Upon comparison of the VP1 sequences of the 15 CV-A6 strains, hardly any variation was observed in the aforementioned regions of VP1 (Supplementary Figure 1), indicating the presence of additional neutralizing epitopes in CV-A6. Another study indicated that the near-atomic-

resolution structure of CV-A6 A-particle complexed with a neutralizing antibody mapped an immune-dominant neutralizing epitope (mAb-1D5) onto the surface loops (BC, EF, HI, and DE loops) of VP1 [63]. We compared the VP1 amino acid sequences of 15 CV-A6 strains with the 12 critical residues in the VP1 epitope of CV-A6 identified by mAb-1D5 [63] and observed a difference (D or N) at 138aa within the DE loop region (Supplementary Table 2). However, this change was not associated with cross-neutralization activity. The binding activities of S112 and S103 were evaluated using five mAbs (1D5 [63], 2B5, 4D6, 7F9, and 6C8) that target the conformational epitopes of CV-A6 with high neutralizing activity. A significant difference in the binding activity to mAb-2B5 was observed between S112 and S103 (*P*

= 0.0066) (Supplementary Figure 2). We also compared the amino acid sequences of VP1, VP2, and VP3 between S112 and S103, which revealed a discrepancy of four amino acids (Supplementary Table 3). The differential sites in the capsid protein may serve as crucial determinants of the disparities in cross-neutralization activity. The epitope of 2B5 remains unclear, and further studies are needed to determine whether it is associated with these four amino acids. The interaction between CV-A6 and the receptor KRMEN1 remains unresolved, leaving uncertainty regarding whether variations in receptor-binding sites contribute to differences in cross-neutralization activity. Further research will be conducted on the structural characteristics of the CV-A6 particles complexed with mAb-2B5 and the receptor KRMEN1 to investigate whether this discovery can explain the variation in cross-neutralization activity among different strains of CV-A6.

In addition, the evaluation of cross-neutralization activity is a pivotal indicator of the broad-spectrum efficacy of vaccine strains [64,65]. During the initial stages of EV-A71 vaccine development, the NIFDC spearheaded cross-neutralization studies of diverse candidate vaccine strains obtained from relevant manufacturers, assisting manufacturers in selecting strains that exhibited exceptional cross-neutralization capabilities across different genotypes and within the same genotype [51,66]. Therefore, an effective EV71 vaccine was successfully developed [43,44,67]. In this study, we collaborated with Chinese CV-A6 vaccine manufacturers to provide *in vitro* and *in vivo* cross-neutralization data, which served as a reference for assessing the broad-spectrum efficacy of the vaccine strains. Fifteen CV-A6 strains were collected and cross-neutralization of CV-A6 strains was conducted to understand the differences in the cross-neutralization ability of different CV-A6 genotypes. The results showed that anti-S110 had good cross-neutralization activity against genotype A and subgenotypes D1 and D3, with the highest GMT (3153.3) and lowest MAX/MIN value (16.0). The anti-S110 serum was diluted 1:48 to investigate its protective effects against lethal challenge in mice. The results showed that anti-S110 serum had good protection against five lethal doses of viruses (including type A and D3 subtypes; the protection rate was 67%–100%), which proved that anti-S110 serum also had broad-spectrum cross-protection ability *in vivo*. Therefore, our study provides valuable reference data for manufacturers regarding the broad-spectrum efficacy of these strains.

Our study provides a framework for selecting detection strains to estimate NtAbs, which is crucial for comparing different vaccines. The cross-neutralization activity data in this study originated primarily from animal models, lacking human vaccination data. Therefore, as the CV-A6 vaccine progresses to clinical trials, we will assess the suitability of the detection strain for NtAb testing in humans.

Disclosure statement

No potential conflict of interest was reported by the author(s).

ORCID

Tong Cheng ⓘ http://orcid.org/0000-0002-1638-6214

References

[1] Repass GL, Palmer WC, Stancamplano FF. Hand, foot, and mouth disease: identifying and managing an acute viral syndrome. Cleve Clin J Med. 2014;81(9):537–543. doi: 10.3949/ccjm.81a.13132

[2] Mirand A, Cohen R, Bisseux M, et al. A large-scale outbreak of hand, foot and mouth disease, France, as at 28 September 2021. Euro Surveill. 2021;26 (43):2100978. doi: 10.2807/1560-7917.ES.2021.26.43.2100978

[3] Carmona RCC, Machado BC, Reis FC, et al. Hand, foot, and mouth disease outbreak by Coxsackievirus A6 during COVID-19 pandemic in 2021, São Paulo, Brazil. J Clin Virol. 2022;154:105245. doi: 10.1016/j.jcv.2022.105245

[4] Kanbayashi D, Kaida A, Hirai Y, et al. An epidemic of hand, foot, and mouth disease caused by coxsackievirus A6 in Osaka city, Japan, in 2017. Jpn J Infect Dis. 2019;72(5):334–336. doi:10.7883/yoken.JJID.2018.532

[5] Lee MHP, Chong YM, Tay CG, et al. Detection of enteroviruses during a 2018 hand, foot and mouth disease outbreak in Malaysia. Trop Biomed. 2021;38 (1):150–153. doi: 10.47665/tb.38.1.026

[6] Nhan LNT, Khanh TH, Hong NTT, et al. Clinical, etiological and epidemiological investigations of hand, foot and mouth disease in southern Vietnam during 2015 – 2018. PLoSNegl Trop Dis. 2020;14(8): e0008544. doi: 10.1371/journal.pntd.0008544

[7] Xu SS, Li HJ, Qiao P, et al. Neonatal hand, foot, and mouth disease due to coxsackievirus A6 in Shanghai. BMC Pediatr. 2020;20(1):364. doi: 10.1186/s12887-020-02262-y

[8] Fonseca MC, Sarmiento L, Resik S, et al. Coxsackievirus A6 and enterovirus 71 causing hand, foot and mouth disease in Cuba, 2011-2013. Arch Virol. 2014;159(9):2451–2455. doi: 10.1007/s00705-014-2071-x

[9] Puenpa J, Chieochansin T, Linsuwanon P, et al. Hand, foot, and mouth disease caused by coxsackievirus A6, Thailand, 2012, Thailand, 2013. Emerg Infect Dis. 2013;19(4):641–643. doi: 10.3201/eid1904.121666

[10] Mirand A, Henquell C, Archimbaud C, et al. Outbreak of hand, foot and mouth disease/herpangina associated with coxsackievirus A6 and A10 infections in 2010, France: a large citywide, prospective observational study. Clin Microbiol Infect. 2012;18(5):E110–E118. doi: 10.1111/j.1469-0691.2012.03789.x

[11] Sinclair C, Gaunt E, Simmonds P, et al. Atypical hand, foot, and mouth disease associated with coxsackievirus A6 infection, Edinburgh, United Kingdom, January to February 2014. Euro Surveill. 2014;19(12):20745. doi: 10.2807/1560-7917.es2014.19.12.20745

[12] Horsten HH, Kemp M, Fischer TK, et al. Atypical Hand, Foot, and mouth disease caused by coxsackievirus A6 in Denmark: A diagnostic mimicker. Acta DermVenereol. 2018;98(3):350–354. doi: 10.2340/00015555-2853

[13] Cisterna DM, Lema CL, Martinez LM, et al. Atypical hand, foot, and mouth disease caused by Coxsackievirus A6 in Argentina in 2015. Rev Argent Microbiol. 2019;51(2):140–143. doi: 10.1016/j.ram.2018.05.003

[14] Neri I, Dondi A, Wollenberg A, et al. Atypical forms of hand, foot, and mouth disease: A prospective study of 47 Italian children. Pediatr Dermatol. 2016;33(4):429–437. doi: 10.1111/pde.12871

[15] Justino MCA, Mesquita DS, Souza MF, et al. Atypical hand-foot-mouth disease in Belém, Amazon region, northern Brazil, with detection of coxsackievirus A6. J Clin Virol. 2020;126:104307. doi: 10.1016/j.jcv.2020.104307

[16] Hayman R, Shepherd M, Tarring C, et al. Outbreak of variant hand-foot-and-mouth disease caused by coxsackievirus A6 in Auckland, New Zealand. Paediatr Child Health. Paediatr Child Health. 2014;50(10):751–755. doi: 10.1111/jpc.12708

[17] Li YN, Xiong TT, Meng Y, et al. Risk factors for severe hand, foot, and mouth disease infected with Coxsackievirus A6: A hospital-based case-control study. J Med Virol. 2020;3144–3150. doi: 10.1002/jmv.26343

[18] Du ZQ, Zhao YL, Luo YJ, et al. Ongoing change of severe hand, foot, and mouth disease pathogens in Yunnan, China, 2012 to 2016. J Med Virol. 2019;91(5):881–885. doi: 10.1002/jmv.25393

[19] Luchs A, Azevedo LS, Souza EV, et al. Coxsackievirus A6 strains causing an outbreak of hand-foot-and-mouth disease in Northeastern Brazil in 2018. Rev Inst Med Trop Sao Paulo. 2022;64:e16. doi: 10.1590/s1678-9946202264016

[20] Centers for Disease Control and Prevention (CDC). Notes from the field: severe hand, foot, and mouth disease associated with coxsackievirus A6 - Alabama, Connecticut, California, and Nevada, November 2011-February 2012. MMWR Morb Mortal Wkly Rep. 2012;61(12):213–214.

[21] Chetrit EB, Well YW, Shulman LM, et al. Coxsackievirus A6-related hand foot and mouth disease: skin manifestations in a cluster of adult patients. J Clin Virol. 2014;59(3):201–203. doi: 10.1016/j.jcv.2013.12.012

[22] Yip CCY, Lau SKP, Woo PCY, et al. Human enterovirus 71 epidemics: what's next?. Emerg Health Threats J. 2013;6:19780. doi: 10.3402/ehtj.v6i0.19780

[23] Chen B, Sumi A, Toyoda S, et al. Time series analysis of reported cases of hand, foot, and mouth disease from 2010 to 2013 in Wuhan, China. BMC Infect Dis. 2015;15:495. doi: 10.1186/s12879-015-1233-0

[24] Puenpa J, Saengdao N, Khanarat N, et al. Evolutionary and genetic recombination analyses of coxsackievirus A6 variants associated with hand, foot, and mouth disease outbreaks in Thailand between 2019 and 2022. Viruses. 2023;15(1):73. doi: 10.3390/v15010073

[25] Yoshitomi H, Ashizuka Y, Ichihara S, et al. Molecular epidemiology of coxsackievirus A6 derived from hand, foot, and mouth disease in Fukuoka between 2013 and 2017. J Med Virol. 2018;90(11):1712–1719. doi: 10.1002/jmv.25250

[26] Kanbayashi D, Kaida A, Hirai Y, et al. An epidemic of hand, foot, and mouth disease caused by coxsackievirus A6 in Osaka city, Japan, in 2017. Jpn J Infect Dis. 2019;72(5):334–336. doi: 10.7883/yoken.JJID.2018.532

[27] Sanjay RE, Josmi J, Sasidharanpillai S, et al. Molecular epidemiology of enteroviruses associated with hand, foot, and mouth disease in South India from 2015 to 2017. Arch Virol. 2022;167(11):2229–2238. doi: 10.1007/s00705-022-05561-0

[28] Wei SH, Huang YP, Liu MC, et al. An outbreak of coxsackievirus A6 hand, foot, and mouth disease associated with onychomadesis in Taiwan, 2010. BMC Infect Dis. 2011;11:346. doi: 10.1186/1471-2334-11-346

[29] Dong SB, Wang XL, Huo D, et al. Epidemiological characteristics of hand, foot and mouth disease among people aged 6 and over in Beijing, 2011-2020. Zhonghua Liu Xing Bing Xue Za Zhi. 2022;43(2):207–212. doi: 10.3760/cma.j.cn112338-20210511-00387

[30] Wang JY, Teng Z, Cui XQ, et al. Epidemiological and serological surveillance of hand-foot-and-mouth disease in Shanghai, China, 2012–2016, People's Republic of China, 2018. Emerg Microbes Infect. 2018;7(1):1. doi: 10.1038/s41426-017-0011-z

[31] Lu J, Zeng H, Zheng H, et al. Hand, foot and mouth disease in Guangdong, China, in 2013: new trends in the continuing epidemic. Clin Microbiol Infect. 2014;20(7):O442–O445. doi: 10.1111/1469-0691.12468

[32] Li JL, Yuan J, Yang F, et al. Epidemic characteristics of hand, foot, and mouth disease in southern China, 2013: coxsackievirus A6 has emerged as the predominant causative agent. J Infect. 2014;69(3):299–303. doi: 10.1016/j.jinf.2014.04.001

[33] Gu HY, Ma CJ, Yang QZ, et al. Hand, foot and mouth disease caused By coxsackievirus A6, Beijing, 2013. Pediatr Infect Dis J. 2014;33(12):1302–1303. doi: 10.1097/INF.0000000000000467

[34] Tan XJ, Li L, Zhang BM, et al. Molecular epidemiology of coxsackievirus A6 associated with outbreaks of hand, foot, and mouth disease in Tianjin, China, in 2013. Arch Virol. 2015;160(4):1097–1104. doi: 10.1007/s00705-015-2340-3

[35] Han JF, Xu S, Zhang Y, et al. Hand, foot and mouth disease outbreak caused by coxsackievirus A6, China 2013. J Infect. 2014;69(3):303–305. doi: 10.1016/j.jinf.2014.03.015

[36] Li JJ, Pan H, Wang XS, et al. Epidemiological surveillance of hand, foot and mouth disease in Shanghai in

2014-2016, prior to the introduction of the enterovirus 71 vaccine. Emerg Microbes Infect. 2018;7(1):1–7. doi: 10.1038/s41426-018-0035-z

[37] Cui Y, Yang YN, Zheng RR, et al. Epidemiological characteristics of hand, foot, and mouth disease clusters during 2016-2020 in Beijing, China. J Med Virol. 2022;94(10):4934–4943. doi: 10.1002/jmv.27906

[38] Duan XX, Zhang CY, Wang X, et al. Molecular epidemiology and clinical features of hand, foot and mouth disease requiring hospitalization after the use of enterovirus A71 inactivated vaccine in Chengdu, People's Republic of China, 2017-2022: a descriptive study. Emerg Microbes Infect. 2022;11(1):2510–2519. doi: 10.1080/22221751.2022.2125346

[39] Cabrerizo M, Tarragó D, Almagro CM, et al. Molecular epidemiology of enterovirus 71, coxsackievirus A16 and A6 associated with hand, foot and mouth disease in Spain. Clin Microbiol Infect. 2014;20(3):O150–O156. doi: 10.1111/1469-0691.12361

[40] Andrés C, Guasch E, Piñana M, et al. Recombinant CV-A6 strains related to hand-foot-mouth disease and herpangina at primary care centers (Barcelona, Spain). Future Microbiol. 2019;14:499–507. doi: 10.2217/fmb-2018-0336

[41] Bujaki E, Farkas A, Rigó Z, et al. Distribution of enterovirus genotypes detected in clinical samples in Hungary, 2010-2018. Acta Microbiol Immunol Hung. 2020;67(4):201–208. doi: 10.1556/030.2020.01200

[42] Han Y, Ji H, Shen WQ, et al. Disease burden in patients with severe hand, foot, and mouth disease in Jiangsu Province: a cross-sectional study. Hum Vaccin Immunother. 2022;18(5):2049168. doi: 10.1080/21645515.2022.2049168

[43] Mao QY, Wang YP, Bian LL, et al. EV71 vaccine, a new tool to control outbreaks of hand, foot and mouth disease (HFMD). Expert Rev Vaccines. 2016;15(5):599–606. doi: 10.1586/14760584.2016.1138862

[44] Mao QY, Wang YP, Bian LL, et al. EV-A71 vaccine licensure: a first step for multivalent enterovirus vaccine to control HFMD and other severe diseases. Emerg Microbes Infect. 2016;5(7):1. doi: 10.1038/emi.2016.73

[45] Zhou Y, Shen CY, Zhang C, et al. Yeast-produced recombinant virus-like particles of coxsackievirus A6 elicited protective antibodies in mice. Antiviral Res. 2016;132:165–169. doi: 10.1016/j.antiviral.2016.06.004

[46] Zhang ZJ, Dong ZP, Wang Q, et al. Characterization of an inactivated whole-virus bivalent vaccine that induces balanced protective immunity against coxsackievirus A6 and A10 in mice. Vaccine. 2018;36(46):7095–7104. doi: 10.1016/j.vaccine.2018.09.069

[47] Caine EA, Fuchs J, Das SC, et al. Efficacy of a trivalent hand, foot, and mouth disease vaccine against enterovirus 71 and coxsackieviruses A16 and A6 in mice. Viruses. 2015;7:5919–5932. doi: 10.3390/v7112916

[48] Liu CC, Guo MS, Rung S, et al. Immunological and biochemical characterizations of coxsackievirus A6 and A10 viral particles. Antiviral Res. 2016;129:58–66. doi: 10.1016/j.antiviral.2016.02.008

[49] Lim H, Ln HJ, Lee JA, et al. The immunogenicity and protection effect of an inactivated coxsackievirus A6, A10, and A16 vaccine against hand, foot, and mouth disease. Vaccine. 2018;36(24):3445–3452. doi: 10.1016/j.vaccine.2018.05.005

[50] Zhang W, Dai WL, Zhang C, et al. A virus-like particle-based tetravalent vaccine for hand, foot, and mouth disease elicits broad and balanced protective immunity. Emerg Microbes Infect. 2018;7(1):1. doi: 10.1038/s41426-018-0094-1

[51] Liu P, Yuan YD, Cui BP, et al. Cross-Antigenicity between EV71 Sub-genotypes: implications for vaccine efficacy. Viruses. 2021;13(5):720. doi: 10.3390/v13050720

[52] Zhu WB, Jin PF, Li JX, et al. Correlates of protection for inactivated enterovirus 71 vaccine: the analysis of immunological surrogate endpoints. Expert Review of Vaccines.2017;16(9): 945–949. doi: 10.1080/14760584.2017.1335603

[53] Klein M, Chong P. Is a multivalent hand, foot, and mouth disease vaccine feasible? Hum Vaccin Immunother. 2015;11(11):2688–2704. doi: 10.1080/21645515.2015.1049780

[54] Li Y, Gao F, Wang YM, et al. Immunogenicity and safety of inactivated enterovirus A71 vaccines in children aged 6-35 months in China: a non-inferiority, randomised controlled trial. Lancet Reg Health West Pac. 2021;16:100284. doi: 10.1016/j.lanwpc.2021.100284

[55] Zhu FC, Xu WB, Xia JL, et al. Efficacy, safety, and immunogenicity of an enterovirus 71 vaccine in China. N Engl J Med. 2014;370:818–828. doi: 10.1056/NEJMoa1304923

[56] Huo YQ, Yang JH, Liu P, et al. Evaluation of the cross-neutralization activities elicited by Coxsackievirus A10 vaccine strains. Hum Vaccin Immunother. 2024;17(12):5334–5347. doi: 10.1080/21645515.2021.1978792

[57] Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. World J Virol. 2016;5(2):85–86. doi: 10.5501/wjv.v5.i2.85

[58] Song Y, Zhang Y, Ji TJ, et al. Persistent circulation of Coxsackievirus A6 of genotype D3 in mainland of China between 2008 and 2015. Sci Rep. 2017;7(1):5491. doi: 10.1038/s41598-017-05618-0

[59] Zhu PY, Ji WQ, Li D, et al. Current status of hand-foot-and-mouth disease. J Biomed Sci. 2023;30(1):15. doi: 10.1186/s12929-023-00908-4

[60] Dalldorf G. The coxsackievirus group. AnnN Y Acad Sci 1953;56(3):583–586. doi: 10.1111/j.1749-6632.1953.tb30251.x

[61] Fu XM, Wan ZZ, Li YP, et al. National epidemiology and evolutionary history of four hand, foot and mouth disease-related enteroviruses in China from 2008 to 2016. Virol Sin. 2020;35(1):21–33. doi: 10.1007/s12250-019-00169-2

[62] Chen JH, Zhang C, Zhou Y, et al. A 3.0-angstrom resolution cryo-electron microscopy structure and antigenic sites of coxsackievirus A6-like particles. J Virol. 2018;92(2):e01257. doi: 10.1128/JVI.01257-17

[63] Zhu R, Yu H, Hong QY, et al. Atomic structures of Coxsackievirus A6 and its complex with a neutralizing antibody. Nat Commun. 2017;8(1):505. doi: 10.1038/s41467-017-00477-9

[64] World Health Organization. Recommendations to assure the quality, safety and 6 efficacy of Enterovirus 71 vaccines (inactivated).[cited 2023 Oct 10]. Available from: https://www.who.int/news-room/articles-detail/call-for-comments-on-ev71-recommendations.

[65] Lei DL, Griffiths E, Martin J. WHO working group meeting to develop WHO Recommendations to assure

the quality, safety and efficacy of enterovirus 71 vaccines. Vaccine. 2020;38(32):4917–4923. doi: 10.1016/j.vaccine.2020.05.001

[66] Mao QY, Li N, Yu X, et al. Antigenicity, animal protective effect and genetic characteristics of candidate vaccine strains of enterovirus 71. Arch Virol. 2012;157(1):37–41. doi: 10.1007/s00705-011-1136-3

[67] Elmgren L, Li XG, Wilson C, et al. A global regulatory science agenda for vaccines. Vaccine. 2013;31(Suppl): B163–B175. doi: 10.1016/j.vaccine.2012.10.117