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RESEARCH ARTICLE

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Establishment of the 1st Chinese national standard for CA6 neutralizing antibody

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ABSTRACT

Coxsackievirus A6 (CA6) is one of the major causative agents of herpangina and hand-foot-mouth disease (HFMD). Since 2008, CA6 has circulated widely around the world. Especially in Asia-Pacific region CA6 had even replaced enterovirus A71 (EV71) and coxsackievirus A16 (CA16) as the main prevalent strain of HFMD. In the recent 10 years, monovalent and multivalent vaccines against CA6 have been researched and developed by manufacturers from China, Korea, and the USA. The neutralizing antibody titer is a key indicator for accurately evaluating immunogenicity of vaccine. However, so far, the World Health Organization international standard for CA6 neutralizing antibody has not been available. In order to meet the needs of evaluating the immunogenicity of vaccines against CA6, the first Chinese national standard for CA6 neutralizing antibody was established, which was conducted to ensure that methods used to measure the neutralizing antibody titers against CA6 are accurate, reliable, and comparable. Three lyophilized candidate standards (29#, 39# and 44#) were produced with 0.40 ml/vial from plasma samples donated by healthy individuals. The collaborative study showed that the 29# candidate standard could effectively minimize the variability in neutralization titers between labs and across challenging viruses of different genotypes (A, D1, and D3). Therefore, the 29# candidate sample was established as the first Chinese national standard for CA6 neutralizing antibody test. This standard has good long-term stability and was assigned a potency of 150 units per milliliter (U/ml) of CA6 neutralizing antibody. It will contribute to ensure uniformity of potency or activity of vaccines and potentially therapeutic antibody preparations.

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Introduction

HFMD is a common infectious disease caused by a variety of enteroviruses (EVs) and is endemic worldwide. Most patients present with hand, foot, and mouth rashes, herpes, and fever that resolve spontaneously without specific intervention.[1] However, some of these patients may develop severe neurological symptoms such as encephalitis, myocarditis, flaccid paralysis, pneumonia, etc.[2–4] The main pathogens of HFMD include coxsackievirus group A (CA) and group B (CB), enterovirus A71 (EV71), and some echoviruses, among which EV71 and CA16 are the predominant pathogens.[5,6]

In recent years, epidemiological data show that the global prevalence of CA6-associated HFMD has increased significantly and has become a major epidemic agent of HFMD in parts of Asia, Europe, and North America, receiving increasing attention.[7–10] In 2008, HFMD outbreak caused by CA6 in Finland, 71% of 117 samples sequenced for VP1 were CA6.[11] CA6 was the predominant strain in the HFMD outbreaks in Spain[12] between 2011 and 2012 and California in the USA[13] in 2012, Edinburgh in the UK[14] in 2013, and France in 2014–2015.[15] Especially in the Asia-Pacific region, outbreaks of HFMD caused by CA6 replacing EV71 and CA16 were reported in Singapore in 2008[16] Taiwan of China in 2009–2010,[17] Japan in 2011,[18] Thailand in 2012,[19] and especially in mainland China between 2011 and 2018 in several places, such as Guangzhou,[20] Shenzhen,[21] Beijing,[22] and Hangzhou.[23]

Vaccine is the most significant mean to prevent and control disease epidemics. Currently, several companies have been developing CA6 monovalent vaccine or CA6-HFMD multivalent vaccines, including inactivated vaccines and virus-like particle (VLP) vaccines, all of which are in preclinical research phases.[24–31] The neutralizing antibody level is a key indicator for the evaluation of vaccine immunogenicity. Currently, the CA6 neutralizing antibody assay is the microcytopathic method, which is recognized as the gold standard for enterovirus neutralizing antibody detection in the world. However, there are many affecting factors about methods, such as long test cycles and lack of reference standards, which make it difficult to compare horizontally the test results among laboratories, and seriously restrict the development and research (R&D) of CA6 vaccine and other related research. Neutralizing antibody standard is essential to ensure the accuracy of test and the comparability of CA6 neutralizing potency assay results, as well as to improve the development of CA6 vaccine. In addition, standard is an indispensable tool for controlling the quality of biological products in drug inspection.

The aim of the study was to establish the first national standard for CA6 neutralizing antibody. The three lyophilizedcandidate standards (29#, 39#, and 44#) of different CA6 neutralizing antibody titers were produced from plasma samples donated by healthy individuals in China. The collaborative

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study was organized by the National Institutes for Food and Drug Control (NIFDC), which is one of the World Health Organization Collaborating Centers (WHO CCs)[32] and one of the National Quality Control Laboratories (NCLs). Other participant labs all have experience in CA6-related research. The establishment of this national standard aimed to standardize the methods for neutralization assays, ensure the accuracy of assays and the comparability of neutralizing antibody titers between different labs and products, and effectively control the quality of vaccines and therapeutics for CA6.

Materials and methods

Selection of candidate standard materials

To prepare the CA6 neutralizing antibody standard candidates, 50 plasma samples (donated by Hualan Biological Engineering, Inc.) from healthy people in Henan province of China were collected, each of which was more than 400 ml. All plasma was tested for CA6 neutralizing antibody, hepatitis B surface antigen (HBsAg), hepatitis C antibody, HIV-1/HIV-2 antibody, and syphilis antibody, respectively. Three plasma samples (29#, 39#, and 44#) that were positive for CA6 neutralizing antibody and negative for HBsAg, HCV antibody, HIV-1/HIV-2 antibody, and syphilis antibody were selected as candidate materials.

Defibrillation of candidate raw materials

After testing the pH and protein content of the candidate plasma, 1.1 ml of 0.125 mol/L $CaCl_2$ (containing 35IU/ml thrombin) was added to 10 ml plasma and rotated the three mixtures slightly in a 37°C water bath for 35 min until fibrin was no longer released. After placing it overnight at 2–8°C, the mixtures were centrifuged at 8000 rpm for 10 min at 2–8°C, then three supernatants were taken to obtain 3 serum candidates (29#, 39#, and 44#).

Filling, freeze-drying, and sealing

According to the relevant requirements for the preparation of standards of WHO guidelines[33,34] and of Chinese Pharmacopoeia (2020 version),[35] three candidate serum standards (29#, 39#, and 44#) of CA6 neutralizing antibodies were filled and lyophilized with 0.40 ml/vial, and then placed at −20°C for a long-term storage. The residual moisture content of lyophilized samples was determined by the Karl Fischer method (Metrohm, 852 Titrando, Switzerland). Neutralizing antibody potency tests were performed on all three samples before and after lyophilization.

Collaborative samples

Human serum samples (candidate standards)

Samples 29#, 39#, and 44# were lyophilized preparations and were distributed by NIFDC (2 samples/laboratory, stored at −20°C). The primary neutralizing antibody potency of CA6 tested by NIFDC was 192, 512, and 128, respectively.

Rat serum samples

Five serum samples (Y1, Y2, Y3, Y4, and Y5) were obtained from rats immunized with CA6 strain (CA6-WH-15). The primary potency of neutralizing antibody tested by NIFDC was 48, 16, 48, 48, and 24, respectively.

Each participant was asked to perform five independent CA6 neutralizing antibody assays for each of the three coded candidate serum samples and no less than three independent CA6 neutralizing antibody assays for rat sera. NIFDC distributed the collaborative samples to collaborating laboratories. All the samples were transported and stored at −20°C.

Collaborative study

Collaborative participants

NIFDC, Wuhan Institute of Biological Products Co., Ltd., Sinovac Life Sciences Co., Ltd., Institute of Medical Biology of the Chinese Academy of Medical Sciences participated in the study. They were referred to by a random code number (Lab1, Lab2, Lab3, and Lab4).

Strains for laboratory testing

(1) The main challenge virus. The CA6 strain of D3 genotype (coded CA6-WH-15, 7.7 $lgCCID_{50}$/ml) was kindly provided by Wuhan Institute of Biological Products Co., Ltd. and distributed by NIFDC as the main challenge strain for neutralizing antibody.
(2) Other virus strains. If participants routinely use other genotype strains in the laboratory, they were encouraged to also evaluate the panel of sera against other CA6 virus strains as well. Thus, in addition to the main CA6 challenge strain, Lab 1-3 also used other four detecting strains, including CA6-Gdula (Genbank no. AY421764.1, 7.8 $lgCCID_{50}$/ml) of A genotype, CA6-XM (Genbank no. KR706309.1, 8.1 $lgCCID_{50}$/ml) of D1 genotype, CA6-17-155 (7.4 $lgCCID_{50}$/ml) of D3 genotype, and CA6-YN129 (7.0 $lgCCID_{50}$/ml) of D3 genotype. Among them, Lab 1 used other two detecting strains.

Laboratory method

Each laboratory used the microcytopathic method (RD cells, purchased from ATCC) and performed independent testing in accordance with the same experimental protocol. The serum samples were diluted at a starting ratio of 1:8. The above serum samples were added to the 96-well plates and then neutralized at 37°C for 2 h after the addition of the CA6 assay strain. The RD cell suspension was prepared at a concentration of 2×10^5 cells/ml, and 0.1 mL of cell suspension was added to each well (including the virus return drop well), mixed well, and incubated in a 5% CO_2 incubator at 35°C. The final result was usually determined in 6–7 days.

Stability studies

The CA6 candidate standards were placed at 37°C for 0, 0.5 and 1 month, 2–8°C for 0, 1, 36, and 12 months and −20°C for 0, 6, 12, 24 and 36 months, respectively. At each time point, two standards were taken to detect the titer of a neutralizing antibody.

Statistical method

For each sample, the end point titers were defined as the dilution rate showing 50% inhibition of the cytopathic effect (CPE). Titers were converted directly into relative potencies by dividing the titer value by that obtained for the appropriate standard. Potencies relative to the three candidate standards, RT29#, RT39#, and RT44#, were calculated relative to the geometric mean (GM) of the results obtained for the 29#, 39#, and 44#. Mean estimates for each sample in each laboratory were taken as GM of three assays performed. Variability in results (end point titer and relative potency) between assays within laboratories and between laboratories was assessed using geometric coefficients of variation (GCV = {10 s-1}×100% where is the standard deviation of the log10 transformed results), within assay variation was assessed using the relative potencies of the three candidate standards 29#, 39#, and 44#. Pooled GCVs across all laboratories were calculated for all samples for end point titers and potencies relative to the candidate standards. Before statistical analysis, the natural logarithm of potencies was transformed to fit a normal distribution.[36] The normal distribution of the data was analyzed using the Shapiro–Wilk (SW) test.

Results

Preliminary characterization and preparation of candidate standards

A total of 50 convalescent human plasmas were initially tested against CA6 strain (CA6-WH-15) by microcytopathic method, and three plasmas (29 #, 39#, and 44#) with CA6 neutralizing antibody titer >1:96 and negative for HBsAg, HCV antibody, HIV-1, and HIV-2 antibody and syphilis antibody were selected. After deliberation, three lyophilized candidate standards were prepared according to the relevant standard-preparation requirements of WHO and Chinese Pharmacopoeia, with 2317 ampoules, 2255 ampoules, and 2397 ampoules, respectively.

Collaborative results for candidate standards

The four laboratories completed five independent CA6 neutralizing antibody tests according to the same experimental protocol, and the results showed that all laboratory data were valid. The CA6 pooled neutralizing antibody geometric mean titers (pooled GMTs) of candidate standards 29#, 39# and 44# were 169.5, 1140.4, and 114.1, respectively, with CV values of 62.7%, 65.1%, and 39.1%, respectively (see Table 1). The frequency distribution of the test results of each laboratory is shown in Figure 1, and the neutralizing antibody titers of the three candidate standards are all in a normal distribution ($p > .05$).

Between-laboratory variability

With reference to the pooled geometric mean titers mentioned above, 29#, 39#, and 44# candidate standards were assigned values at 150 U/ml, 1000 U/ml, and 100 U/ml, respectively, which were used to calculate the relative potency results of all samples.

Table 1. Results of collaborative calibration of neutralizing antibody titers for CA6 standards (titer).

		Candidate standard		
Laboratory	Detection times	29#	39#	44#
1	1	512	2048	192
	2	256	2048	64
	3	96	1024	128
	4	192	2048	128
	5	384	2048	96
	GM	**247.5**	**1782.9**	**114.1**
	GCV (%)	**91.3**	**36.3**	**50.2**
2	1	256	4096	192
	2	128	768	48
	3	96	768	64
	4	64	1024	96
	5	192	1536	48
	GM	**131.1**	**1306.0**	**77.1**
	GCV (%)	**73.1**	**101.2**	**79.3**
3	1	64	512	128
	2	64	512	128
	3	128	768	64
	4	128	512	128
	5	128	512	128
	GM	**97.0**	**555.3**	**111.4**
	GCV (%)	**46.2**	**19.9**	**36.3**
4	1	768	1536	128
	2	256	768	128
	3	128	1024	96
	4	512	3096	256
	5	96	1024	384
	GM	**262.1**	**1308.1**	**172.9**
	GCV (%)	**142.1**	**71.8**	**77.5**
Pooled GM		**169.5**	**1140.4**	**114.1**
GCV(%)		**62.7**	**65.1**	**39.1**

Three human serum samples and five animal serum samples were tested with the same challenge strain, CA6-WH-15 strain of D3 genotype (Table 2). The results showed that the mean endpoint titer GMTs of human sera (i.e. candidate standards) was 280.4, and the mean GCV was 55.6%. After using 150 U/ml, 1000 U/ml, and 100 U/ml of 29#, 39# and 44# to calculate the relative titers, the mean GMTs of RP to 29#, 39# and 44# were 319.3 U/ml, 121.9 U/ml, and 385.3 U/ml with the mean GCV of 41.2%, 57.8%, and 63.7%, respectively. Congruously, the mean endpoint titer GMTs of animal sera between laboratories was 30.9, and the mean GCV was 51.0%. After using 150 U/ml, 1000 U/ml, and 100 U/ml of 29#, 39#, and 44# to calculate the relative titers, the mean GMTs of RP to 29#, 39# and 44# were 27.1, 27.3, and 27.6 with mean GCVs of 30.2%, 31.9%, and 43.8%, respectively. The results showed that when 29# was used as the standard to calculate the relative titer, the difference of GCV between the lab detection for human sera and for animal sera had the downward trend.

Comparison of different challenge viruses

For three human sera (i.e. candidate standard), the mean GCV of the endpoint titer in Lab1, Lab2, and Lab3 were 177.6%, 671.5%, and 556.7%, respectively (Table 3). When using 29# of 150 U/ml to calculate the relative titer, the mean GCVs of RP to 29# in three laboratories were 25.1%, 181.1%, and 80.5%, respectively. When using 39# of 1000 U/ml to calculate the relative titer, the mean GCVs of RP to 39# in three laboratories were 27.9%, 447.8%, and 357.0%, respectively. When the relative titer was calculated using

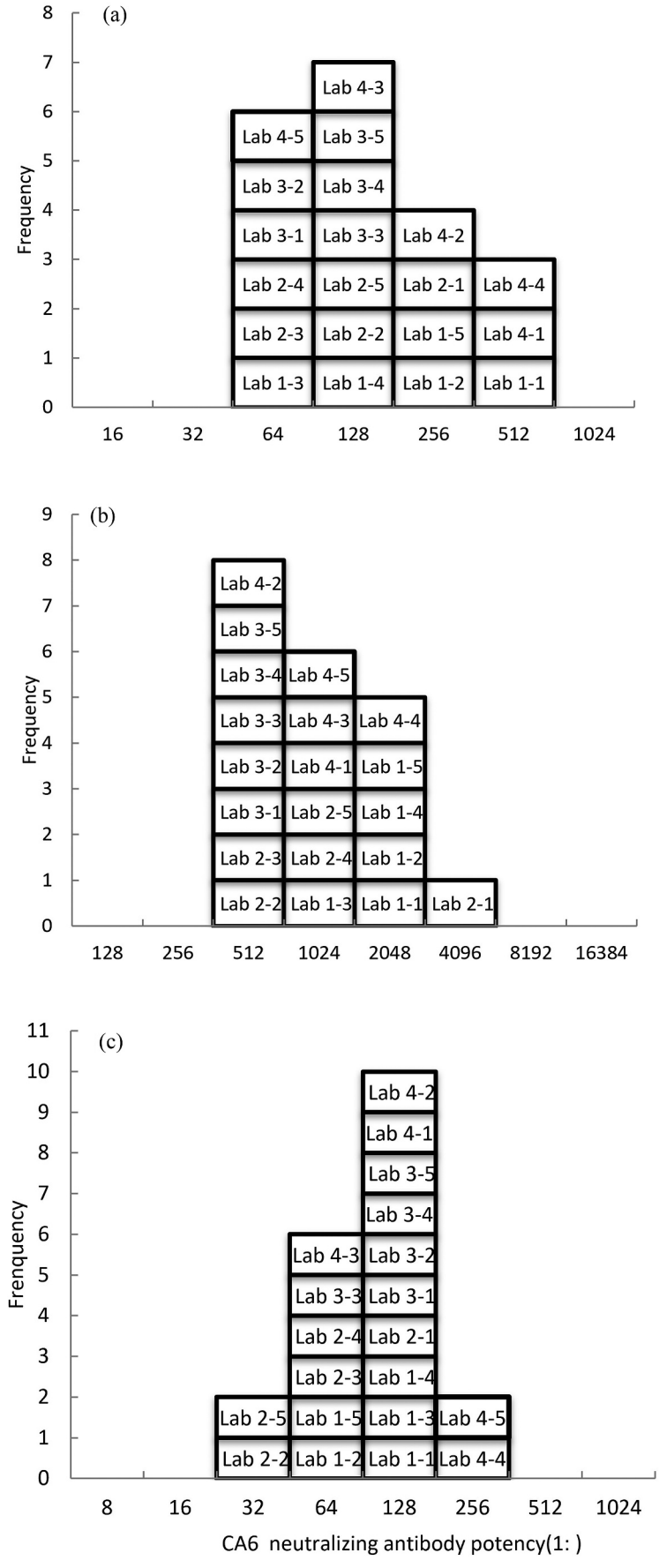

Figure 1. Frequency distribution of collaborative calibration of CA6 candidate standards. (a) is the frequency distribution from 4 collaborative labs of 29# candidate standard; (b) is the frequency distribution from 4 collaborative labs of 39# candidate standard; (c) is the frequency distribution from 4 collaborative labs of 44# candidate standard.

Table 2. Geometric mean potencies and relative values for samples tested with the same challenge virus (CA6-WH-15 of D3 genotype).

		Samples									
		Human serum				Rat serum					
	Results	29#	39#	44#	Mean	Y1	Y2	Y3	Y4	Y5	Mean
End Point Titer	GM	169.5	1140.4	114.1	**280.4**	45.7	27.1	17.8	41.3	40.0	**30.9**
	GCV (%)	62.7	65.1	39.1	**55.6**	35.9	30.3	80.0	51.6	21.4	**51.0**
Relative to 29#	GM	/	1009.5	101.0	**319.3**	40.2	23.8	15.6	36.3	35.2	**27.1**
	GCV (%)	/	35.3	47.1	**41.2**	28.7	45.1	44.8	27.2	18.5	**30.2**
Relative to 39#	GM	148.6	/	100.0	**121.9**	40.4	24.0	15.7	36.6	35.4	**27.3**
	GCV (%)	35.3	/	80.2	**57.8**	19.9	88.8	37.5	18.2	40.0	**31.9**
Relative to 44#	GM	148.5	999.6	/	**385.3**	40.8	24.2	15.9	36.9	35.7	**27.6**
	GCV (%)	47.1	80.2	/	**63.7**	43.5	34.2	61.1	45.2	25.2	**43.8**

"/" is NA, and relative titers cannot be calculated for self-titers.

44# of 100 U/ml, the mean GCVs of RP to 44# in three laboratories were 17.8%, 391.1%, and 197.3%, respectively.

For five animal sera, the mean GCVs of the endpoint titer in Lab1, Lab2, and Lab3 were 104.6%, 241.2%, and 111.1%, respectively (Table 3). When using 29# of 150 U/ml to calculate the relative titers, the mean GCVs of RP to 29# in the three laboratories were 32.0%, 51.0%, and 77.6%, respectively. When using 39# of 1000 U/ml to calculate the relative titers, the mean GCVs of RP to 39# in three laboratories were 55.9%, 409.7%, and 602.5%, respectively. When using 44# of 100 U/ml to calculate the relative titer, the mean GCVs of RP to 44# in the three laboratories were 58.2%, 59.6%, and 26.3%, respectively. It is suggested that the three candidate standards can significantly reduce the detection difference between different challenge strains.

According to Tables 2 and 3, when the same challenge strain was used, 29# can reduce the inter-laboratory mean GCV (endpoint titer) of human sera and animal sera from 55.6% to 41.2% and from 51.0% to 30.2%, respectively. When using different strains in Lab1, Lab2, and Lab3, 29# can reduce the inter-strain mean GCVs of human sera from 177.6% (Lab1), 671.5% (Lab2), and 556.7% (Lab3) to 25.1% (Lab1), 181.1% (Lab2), and 80.5% (Lab3), and can reduce the mean GCVs of animal sera from 104.6% (Lab1), 241.2% (Lab2), and 111.1% (Lab3) to 32.0% (Lab1), 51.0% (Lab2), and 77.6% (Lab3). The data suggested that the 29# candidate standard can effectively reduce the detection differences between laboratories and strains. Therefore, it is recommended 29# as the first Chinese national standard for testing CA6 neutralizing antibody and assigned value of 150 units per milliliter (U/ml).

Stability study

The 29# CA6 candidate standard was placed at 37°C for 0, 0.5 and 1 month, at 2–8°C for 0, 1, 3, 6 and 12 months, and at −20°C for 0, 6, 12, 24 and 36 months (Table 4). Two standard samples were taken at each time point to detect the neutralizing antibody titer. The results showed that with 0 days as the control, CA6 GMT did not decrease significantly after being placed at 37°C for 1 month, at 2–8°C for 12 months, and at −20°C for 3 years ($p > .05$).

Discussion

HFMD is a serious disease endangering the health of children around the world. The main pathogens causing HFMD are complex and diverse, including EV71, CA16, and CA6. In the end of 2015, EV71 inactivated vaccine was approved for marketing in China, which filled the vacancy of HFMD vaccine.[37] However, there is no cross-immunity protection between different HFMD pathogens. In recent years, CA6 has even replaced EV71 as the main epidemic strain of HFMD in some areas.[38–45] Therefore, more and more companies and research institutions are paying attention to R&D of CA6-related vaccines. Researchers from at least eight institutions mainly in the Asia-Pacific region have carried out R&D of CA6 monovalent or multivalent vaccines, including inactivated vaccines, VLP vaccines, and subunit vaccines.[24–31]

Neutralizing antibody is an important indicator for evaluating the immunogenicity of vaccines. Meanwhile, reliable test results are of great significance to improve progress of vaccine development. At present, most of the neutralizing antibody

Table 3. GCV% of geometric mean potencies and relative values for samples tested with the different challenge viruses.

		Samples									
		Human serum				Animal serum					
Labs	Results	29#	39#	44#	Mean	Y1	Y2	Y3	Y4	Y5	Mean
Lab1 (challenge viruses :CA6-WH-15, CA6-Gdula, CA6-XM)	End Point Titer GCV%	186.8	181.3	164.6	**177.6**	111.4	137.0	143.2	88.4	82.3	**104.6**
	Relative to 29# GCV%	/	35.2	15.0	**25.1**	16.9	169.3	15.8	32.4	47.7	**32.0**
	Relative to 39# GCV%	35.2	/	20.5	**27.9**	32.4	221.6	36.4	55.8	75.6	**55.9**
	Relative to 44# GCV%	15.0	20.5	/	**17.8**	44.4	209.8	31.8	63.3	79.4	**58.2**
Lab2 (challenge viruses :CA6-WH-15, CA6-17-155)	End Point Titer GCV%	379.9	1520.6	113.9	**671.5**	388.0	249.1	132.0	249.1	342.4	**241.2**
	Relative to 29# GCV%	/	237.7	124.4	**181.1**	1.7	37.5	106.9	37.5	8.5	**51.0**
	Relative to 39# GCV%	237.7	/	657.8	**447.8**	232.1	364.2	598.6	364.2	266.3	**409.7**
	Relative to 44# GCV%	124.4	657.8	/	**391.1**	128.2	63.3	8.5	63.3	106.9	**59.6**
Lab3 (challenge viruses :CA6-WH-15, CA6-YN129)	End Point Titer GCV%	137.6	933.9	598.6	**556.7**	306.6	335.1	79.6	111.5	142.2	**111.1**
	Relative to 29# GCV%	/	115.4	45.6	**80.5**	10.0	17.7	105.7	74.7	52.5	**77.6**
	Relative to 39# GCV%	582.0	/	132.0	**357.0**	259.4	235.8	713.5	590.9	503.2	**602.5**
	Relative to 44# GCV%	11.1	383.5	/	**197.3**	126.3	142.2	0.0	17.7	34.8	**26.3**

"/" is NA, and relative titers cannot be calculated for self-titers.

Table 4. Stability study results of 29# candidate standard (GM Titers).

T (℃)/Time (Month)	0 M	0.5 M	1 M	3 M	6 M	12 M	24 M	36 M
37°C	192.0	221.7	221.7	/	/	/	/	/
2–8°C	271.5	/	256.0	156.8	221.7	181.0	/	/
−20°C	271.5	/	/	/	181.0	313.5	192.0	271.5

"/"is NA, this time point did not set samples.

detection methods use the CPE, which is an internationally recognized gold standard. However, it needs to use active substances such as cells and viruses, which always generate great variation to the method. At present, anti-CA6 international standards are not available. In order to promote the R&D progress of CA6 vaccine, this study established the world's first national standard for testing CA6 neutralizing antibody. According to the results of the collaborative study, the standard was assigned a value of 150 U/ml with the qualified homogeneity and residual moisture. The long-term stability of it was good, for the neutralizing antibody titers did not drop significantly, after #29 being placed at −20°C for 36 months. Finally, it has been approved by the Sub-Committee on Biological Products Reference Materials of NIFDC with the code number of 300034. This standard will contribute to the standardized assessment of CA6 vaccine and therapeutics.

This collaborative study organized four experienced institutes to test 3 lyophilized candidate standards (29#, 39#, and 44#) and 5 rat serum samples with the same experimental protocol. The results showed that when using the same experimental protocol but detected by different-genotype strains, great differences in the detection were found. For human serum, the mean GCVs of the endpoint titer of different strains could reach 177.6%–671.5%. After standardization by 29# standard, the mean GCVs can be significantly reduced to 25.1%–181.1%. For animal serum, the mean GCVs of the endpoint titer of different strains could reach 104.6–241.2%. After standardization by 29# standard, the mean GCVs can be significantly reduced to 32.0–77.6%. When the same protocol and the testing strain were used for detection, the GCVs between laboratories is smaller, and the GCVs of mean endpoint titers of human sera and rat sera were 55.6% and 51.0%, respectively, which could be further reduced to 41.2% and 30.2% after standardized by the 29# standard. It was shown that the 29# standard could significantly reduce the detection variation between strains and laboratories. The results showed that when the experimental protocol was unified, different challenge strains may be the main factors affecting the detection results of CA6 neutralizing antibody. Enteroviruses, as RNA viruses, are prone to mutation or recombination. The prevalent strains of CA6 in the world are mainly D genotype, which is divided into three subtypes of D1-D3 in the evolutionary tree. After 2008, the prevalent strains in the world mainly belong to the D3 genotype.[46] Therefore, this collaborative study used the D3-genotype strain (code: CA6-WH -15) as the main challenge strain and also used other strains of different genotypes including A genotype (CA6-Gdula), D1 genotype (CA6-XM), and D3 genotypes (CA6-17-155 and CA6-YN129). In the future, we should constantly pay attention to the applicability of this standard to ensure that this standard could be suitable to the newly appearing mutant strains.

China is the most populous country in the world, in which many vaccines are developed and used with the characteristics of many kinds of vaccines, many vaccine manufacturers, and the huge consumptions of vaccines. Therefore, as one of WHO CCs and one of NCLs, NIFDC have rich experience in reference-standard research, especially in the development of reference standards for HFMD vaccines. For example, we have established a series of enterovirus related international and national standards, including two EV71 WHO IS in cooperation with National institute for biological standards and control (NIBSC) (Antigen ISwith the code number of 18/116 and assigned value of 14500 IU/ml, antibody IS with the code number of 14/140 and assigned value of 1000IU/ml),[47–49] three EV71 national standards for antigen, antibody, and potency (with the code number of 300016, 300017, and 300020, respectively), and two CA16 national standards for antigen and antibody (with the code number of 300019 and 300030, respectively).[50–53] The successful establishment of the above-mentioned international and national standards plays a key role in the quality control of HFMD vaccines and the related therapeutic researches.

The CA6 neutralizing antibody national standard established in this study is one part of a series of enterovirus standards, which fills the international and domestic vacancies and is of great significance to the development and quality control of CA6 vaccines. This CA6 national standard can standardize the methods for neutralization assays, ensure the accuracy of tests and the comparability of neutralizing antibody titers between different labs and products, and effectively control the quality of vaccines and therapeutics for CA6. For moreover, this standard can not only be used for R&D of vaccine in China but also can promote R&D of vaccine in the entire Western Pacific region, thereby enhancing the prevention and control capability of national or regional HFMD, and effectively protecting infants and young children from the threat of HFMD disease.

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Disclosure statement

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