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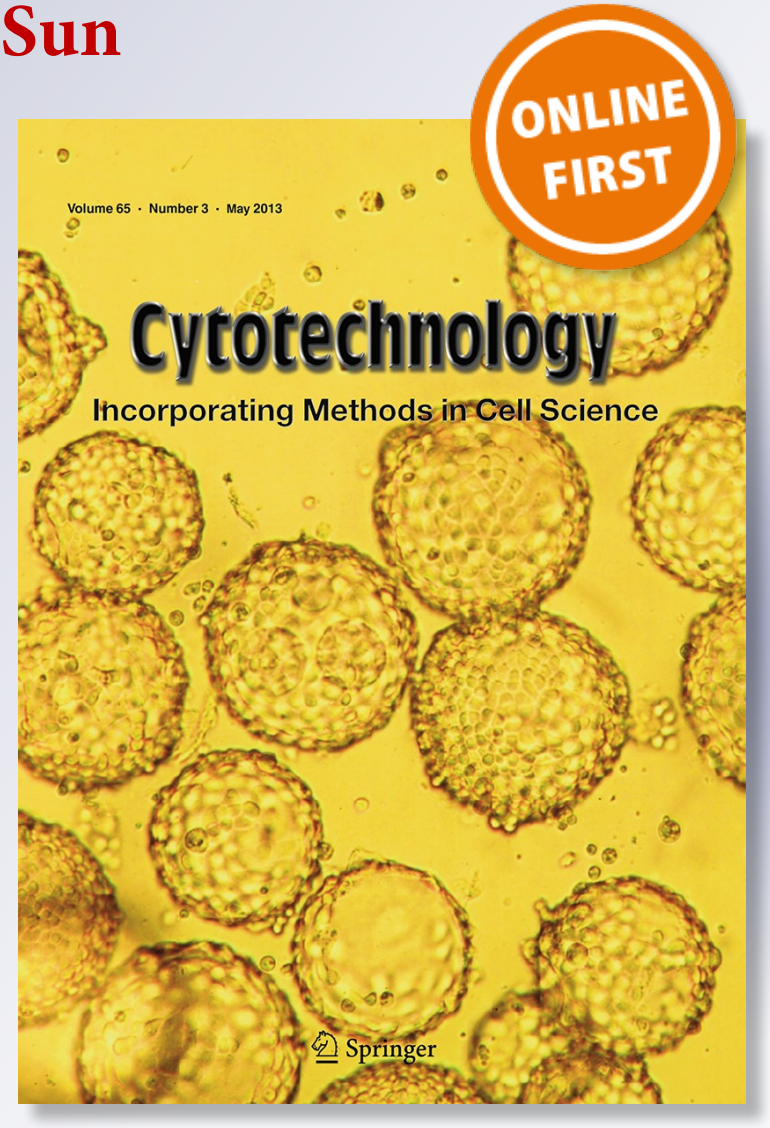

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ORIGINAL RESEARCH

Development of a sandwich ELISA for the quantification of enterovirus 71

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Abstract Since 2008, enterovirus 71 (EV71) has been responsible for high-mortality seasonal epidemics of hand, foot and mouth disease in China. Currently many groups in the world are in the process of developing EV71 vaccines to combat this deadly disease. We have developed three EV71-specific monoclonal antibodies, and in this study we report the establishment of a fast and cost-effective sandwich ELISA kit for measurement of virus concentration in EV71 vaccines using a pair of mouse anti-EV71 monoclonal antibodies. The system is specific for EV71 virus, with no cross-reactivity to coxsackievirus A16, H1N1, rabies, and hepatitis A. Using a reference EV71 vaccine standard, the sensitivity of the assay kit was determined to be 0.82 U/ml, with a linear range between 3.75 and 120 U/ml.

Keywords Enterovirus (EV) 71 · Vaccine · Sandwich ELISA · Quantification

Introduction

Enterovirus 71 (EV71) is one of the major causes of hand, foot and mouth disease (HFMD). Since 2008, viral outbreaks resulting in high mortality have been seen in seasonal epidemics in China and many other parts of Asia (Xu WB. 2009; Kim 2009; Chua and Kasri 2011; Tan et al. 2011). In mainland China, through June 2012, 1.27 million cases of HFMD with 356 deaths have been reported in China (Infectious Case Report for July 2012). Currently there are no effective preventive measures to combat this deadly disease. EV71 represents a huge challenge to public health, and it is extremely critical to develop a safe and effective EV71 vaccine as quickly as possible. Currently many groups, including several from China, are in the process of developing EV71 vaccines based on inactivated virus (Liu et al. 2007; Ong et al. 2010; Mao et al. 2011,2012; Dong et al. 2011; Li et al. 2012).

Antigen content is one of the key parameters for evaluation of active components in the vaccine preparations and must be accurately determined in

Shujun Ma and Qunying Mao have contributed equally to this article.

S. Ma · C. Zhang · W. Yang · Z. Sun · H. Zhang · L. Sun (✉)
AbMax Biotechnology Co., LTD, 99 Kechuang 14th St,BDA, Beijing 101111, China
e-mail: Lsun@antibodychina.com

Q. Mao · Z. Liang
National Institutes for Food and Drug Control, Beijing 100050, China

X. Shen
Beijing Vigoo Biologicals Co., LTD, 4 South San Jian Fan, Beijing 100024, China

S. Bi
Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100052, China

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all finished vaccine products. It is of importance to develop a fast and easy assay for vaccine manufacturers to monitor the production process and for governmental agencies to evaluate the quality of the final vaccine products.

In this study, using a pair of EV71-specific mouse monoclonal antibodies previously developed by our group (Li et al. 2009), we report a simple and cost-effective sandwich ELISA kit for the quantification of EV71 virus in the EV71 vaccine. The specificity of the kit was tested and no cross-reactivity was observed against coxsackievirus A16 (CA16), H1N1, rabies and Hepatitis A viruses. Most importantly, the assay kit has been used by more than 20 EV71 vaccine developers in three different countries, and one of them has entered human phase III clinical study.

Materials and methods

The EV71 vaccine standard and other materials

The EV71 vaccine standard containing formaldehyde-inactivated EV71 C4 subtype virus (1,600 U/ml, 1U = 2 ng total viral protein) was developed by the Chinese National Institutes for Food and Drug Control (Liang et al. 2011). Inactivated CA16 virus and H1N1 VLP were obtained from the Institute for Viral Disease Control and Prevention, Chinese CDC. Inactivated rabies virus was provided by the Jilin University. Hepatitis A virus (HAV) vaccine was purchased from Merck & Co., Inc (Whitehouse Station, NJ, USA). Horseradish peroxidase (HRP) conjugation kit was purchased from Pierce (Rockford, IL, USA).

Anti-EV71 monoclonal antibodies (mAb) and HRP conjugates

The generation of mouse monoclonal antibodies to EV71 was reported previously by our group (Li et al. 2009). The monoclonal antibodies were produced in stationary bioreactors and affinity-purified using protein-G agarose columns (Upstate Biotechnology, Lake Placid, NY, USA). The concentrations of purified IgG were determined by absorbance at OD280. The antibodies were conjugated with horseradish peroxidase according to the manufacturer's instructions. The specific activities of the conjugated antibodies were determined by the ratio OD430/OD280.

Sandwich ELISA

Each well of 96-well high-binding ELISA plates (Corning, Corning, NY, USA) was coated with different capture monoclonal antibodies (mAbs) overnight at 4 °C in 0.05 M Na_2CO_3, pH 9.6. After two washes with PBS, the plates were blocked with blocking reagents (3 % BSA (bovine serum albumin) in PBS), then air-dried and sealed in a plastic bag. Antigen samples were diluted in 3 % BSA-PBS and incubated in the wells for 1 h at room temperature. After two washes, wells were probed with the second HRP-conjugated mAb diluted in 3 % BSA-PBS for another hour. After five washes with PBS containing Tween 20, 0.1 %, (PBST), chromogenic HRP substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added to the wells. The reaction was stopped with Stop solution (0.1 M H_2SO_4) after 30 min incubation at room temperature and absorbance was measured at 450 nm with a plate reader.

Evaluation of the validation parameters of the sandwich ELISA

Specificity: Specificity was determined by testing against EV71 Antigen standard, inactivated CA16 virus, H1N1 VLP, inactivated Rabies virus, and HAV vaccine.

Limit of Detection (LOD): The LOD was examined by analyzing the absorbance values of a blank sample (PBS) in replicates. The LOD was defined as being three times the standard deviation.

Linearity: It was determined by using the linear correlation coefficient of the standard curves established with the reference vaccine standard. The concentrations of the vaccine references were 0, 3.75, 7.5, 15, 30, 60, 120 U/ml. Each point was done in triplicate. The linearity was calculated from the linear regression of the results of the standard curve.

Precision: It was examined for both intra-assay and inter-assay precision. Vaccine samples with high, medium and low contents were used and examined against the standard curve. To asses inter-assay precision, the ELISA was carried out by more than one technician. The standard deviation was calculated. The coefficient of variation (CV) could not be higher

than 10 %, whereas the CV of the inter-assay precision could not be over 20 %.

Results

Monoclonal antibody pairing

Three mouse monoclonal antibodies to EV71, clones 22A12, 13D7 and 1H1, were used for antibody pairing as capturing and/or detection antibodies. Each well was coated with different mAbs at 2 μg/ml, then paired with HRP-conjugated mAbs at 1 μg/ml for detection of EV71 vaccine standards at (25 or 100 U/ml) as shown in the Table 1. Out of the six combinations, it was found that the best combination was mAb 22A12 as capturing antibody and HRP-conjugated mAb 1H1 as detection antibody. In addition, pairing between mAbs 22A12 and 13D7 was also effective, although less.

Optimization of sandwich ELISA

For production of the assay kit, anti-EV71 mAb 22A12 was used as the capture antibody and HRP-conjugated mAb 1H1 was used as the detection antibody. To optimize the production of the kit, two key parameters were tested: (1) concentration of capturing mAb 22A12, and (2) concentration of the detecting mAb 1H1-HRP-conjugate.

Plates were coated with mAb 22A12 at five concentrations (0.5, 1, 2, 4 and 8 μg/ml) in 0.05 M Na_2CO_3, pH 9.6, overnight at 4 °C. After washing, the plates were blocked with 3 % BSA in PBS, and then air-dried. Two different concentrations of vaccine standards were added into each well of the plate, detected with 1 μg/ml of HRP-conjugated mAb 1H1. As shown in Table 2, the positive antigen signal increased as the coating concentration of mAb 22A12 increased from 0.5 to 2 μg/ml. Coating concentrations higher than 2 μg/ml did not increase the signal

Table 1 Anti-EV71 monoclonal antibody pairings

Coating 2 μg/ml	22A12[a]		1H1		13D7	
Enzyme conjugates 1 μg/ml	1H1-HRP[a]	13D7-HRP	22A12-HRP	13D7-HRP	22A12-HRP	1H1-HRP
Vaccine 100 U/ml	1.217 ± 0.036	0.522 ± 0.024	0.988 ± 0.070	0.385 ± 0.008	0.927 ± 0.032	0.629 ± 0.027
Vaccine 25 U/ml	0.374 ± 0.010	0.192 ± 0.007	0.489 ± 0.020	0.232 ± 0.012	0.520 ± 0.022	0.323 ± 0.024
Vaccine 0 U/ml	0.072 ± 0.004	0.066 ± 0.003	0.235 ± 0.012	0.164 ± 0.007	0.248 ± 0.015	0.127 ± 0.018

The values are expressed as mean ± SD

Each well was coated with different capture mAbs overnight at 4 °C in 0.05 M Na_2CO_3, pH 9.6, then blocked with 3 % BSA-PBS. Vaccine standards were diluted in 3 % BSA-PBS and incubated in the wells for 1 h at room temperature. After two washes, wells were probed with HRP-conjugated detection mAbs for another hour. HRP substrate TMB was added to develop the color. At the end of 30 min, absorbance was determined at 450 nm with a plate reader

[a] The best group: 22A12 as capturing antibody and HRP-conjugated 1H1 as detection antibody

Table 2 Effects of coating antibody concentrations

Vaccine (U/ml)	Coating 22A12 (μg/ml)				
	0.5	1	2	4	8
100	0.812 ± 0.004	1.058 ± 0.056	1.203 ± 0.007	1.221 ± 0.006	1.208 ± 0.007
25	0.248 ± 0.003	0.362 ± 0.014	0.381 ± 0.007	0.455 ± 0.024	0.490 ± 0.005
0	0.054 ± 0.002	0.063 ± 0.002	0.07 ± 0.002	0.132 ± 0.005	0.180 ± 0.006

The values are expressed as mean ± SD

Each well was coated with different concentrations of mAb 22A12 overnight at 4 °C in 0.05 M Na_2CO_3, pH9.6. After two washes with PBS, the plates were blocked 3 % BSA-PBS, then air-dried and sealed in a plastic bag. Antigen samples were diluted in 3 % BSA-PBS and incubated in the wells for 1 h at room temperature. After two washes, wells were probed with 1 μg/ml of HRP-conjugated detection mAb 1H1 diluted in 3 % BSA-PBS for another hour. After five washes with PBST, HRP substrate TMB solution was added to the wells. The reaction was stopped after 30 min and the absorbence was measured at 450 nm with a plate reader

Table 3 Effects of detecting antibody concentrations

Vaccine (U/ml)	HRP conjugated-1H1(μg/ml)				
	0.25	0.5	1	2	4
100	0.764 ± 0.008	1.059 ± 0.046	1.223 ± 0.016	1.322 ± 0.008	1.290 ± 0.012
25	0.225 ± 0.012	0.301 ± 0.003	0.358 ± 0.011	0.397 ± 0.008	0.422 ± 0.011
0	0.057 ± 0.002	0.060 ± 0.001	0.074 ± 0.006	0.101 ± 0.009	0.216 ± 0.015

The values are expressed as mean ± SD

Each well was coated with 2 μg/ml of mAb 22A12. Antigen samples were diluted in 3 % BSA-PBS and incubated in the wells for 1 h at room temperature. After two washes, wells were probed with different concentrations of HRP-conjugated detection mAb 1H1 for another hour. After five washes with PBST, HRP substrate TMB solution was added to the wells. The reaction was stopped after 30 min and the absorbence measured at 450 nm with a plate reader

Table 4 Specificity of the sandwich ELISA

Samples	Conc. (ng/ml)	Value
EV71	250	1.321 ± 0.042
	62.5	0.374 ± 0.018
CA16	1,000	0.085 ± 0.006
	100	0.086 ± 0.001
H1N1	1,000	0.082 ± 0.001
	100	0.072 ± 0.001
Rabies Virus	1,000	0.077 ± 0.004
	100	0.077 ± 0.006
Hepatitis A Virus	1,000	0.076 ± 0.005
	100	0.062 ± 0.005
Negative control, 3 %BSA	0	0.061 ± 0.004

The values are expressed as mean ± SD

Two different concentrations for each antigen sample were diluted in 3 % BSA-PBS and tested in triplicate. After two washes, wells were probed with HRP-conjugated detection mAb 1H1 at 1 μg/ml for another hour. After five washes with PBST, HRP substrate TMB solution was added to the wells. The reaction was stopped after 30 min and the absorbence measured at 450 nm with a plate reader

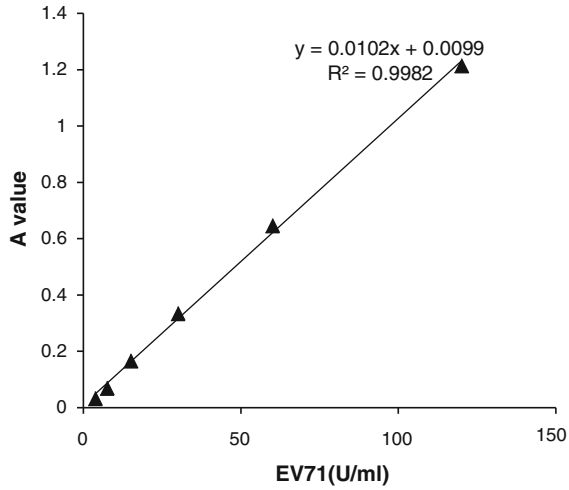

Fig. 1 Standard curve of EV71 sandwich ELISA. Each well was coated with 2 μg/ml of mAb 22A12, blocked with 3 % BSA-PBS, then air-dried. Different concentrations (0, 3.75, 7.5, 15, 30,60, and 120 U/ml) of the EV71 vaccine standards were diluted in 3 % BSA-PBS and tested in triplicate. The LOD was defined as being the mean of the negative control plus two times the standard deviation

significantly, but raised the background. Based on the results, the optimal coating concentration was chosen to be 2 μg/ml.

Five Concentrations of HRP-conjugated mAb 1H1 (0.25, 0.5, 1, 2 and 4 μg/ml) were tested with the plates coated with mAb 22A12 at 2 μg/ml. Results shown in Table 3 demonstrate that the assay gave the best positive signal with reasonable background at 1 to 2 μg/ml of the detecting HRP-conjugated mAb 1H1. Higher antibody concentration only increased the background.

Validations of the EV71 sandwich ELISA

The specificity of the ELISA was determined by testing against inactivated HFMD CA16 virus, inactivated HAV virus, inactivated rabies virus and H1N1 VLP. Two concentrations of each antigen were tested; results are shown in Table 4. No cross-reactivity with CA16, the closest family member of EV71, was observed, indicating that the sandwich ELISA kit we developed is very specific for EV71 virus.

To determine the LOD and the linear range of the sandwich ELISA kit, vaccine standards were diluted to

Table 5 Precision of the assays

	Repeatability (intra-assay)		Intermediate precision (inter-assay)	
	SD (μ/ml)	CV[a] (%)	SD (μ/ml)	CV (%)
100U/ml	3.966	4.10	3.986	4.09
50U/ml	2.361	4.84	2.27	4.64
25U/ml	1.467	5.46	1.478	5.34

Each well was coated with 2 μg/ml of mAb 22A12, blocked with 3 % BSA-PBS, then air-dried. Vaccine samples (100, 50 and 25 U/ml) were used and examined against the standard curve. The precision study was divided in repeatability (intra-assay) and intermediate (inter-assay) precision. To asses inter-assay precision, the ELISA was carried out by more than one technician

[a] Coefficient of variation = (standard deviation [SD]/mean) × 100

0, 3.75, 7.5, 15, 30, 60 and 120 U/ml and measured in triplicate. As shown in Fig. 1, the linear range was between 3.75 and 120 U/ml, with the value of the correlation coefficients of the linear regression (R^2) above 0.99. The LOD was determined to be 0.82 U/ml. Since the concentrations of most of the EV71 vaccine final product will be around 1,600 U/ml, the sandwich ELISA kit should meet the needs for both vaccine manufacturers and governmental agencies.

To asses inter-assay precision, the ELISA was carried out by more than one technician. The standard deviation was calculated. As shown in Table 5, the coefficient of variation (CV) of intra-assay was 4.10–5.46 %, less than 10 %, whereas the CV of the inter-assay precision was 4.09–5.34 %.

Discussion

The ELISA was designed to help the vaccine developers to determine the expression levels of virus at the early development stages, for quality control of the manufacture process and allow the governmental agencies to verify the EV71 content of EV71 vaccines from different manufacturers. In comparison with the in vivo potency evaluation method, this ELISA will reduce the large number of animals to be used, save time and costs.

Most of the EV71 vaccine final products developed have a final concentration of EV71 at 1,600 U/ml, a simple 20-fold dilution of the vaccine with 3 % BSA-PBS will fall into the linear range (between 3.75 and 120 U/ml) of this ELISA kit, making it very user-friendly.

Several vaccine manufacturers are also working on CA16 virus and intend to develop an EV71-CA16 bivalent HDFM vaccine. Polyclonal antibodies raised against inactivated EV71 showed more than 70 % cross-reactivity to CA16 (data not reported here). The in vivo animal study will not be able to measure the exact concentrations of each virus in the finished vaccine product. All other anti-EV71 monoclonal antibodies commercially available were developed against entire EV71 virus and showed strong cross-reactivity with other members of Enterovirus (reported on their data sheets). In this report, we used anti-EV71 mAb 22A12 which was raised against an EV71-specific sequence (amino acids 208–222 of VP1 from EV71 which is not shared with any other known members of Enterovirus). We reported here that this ELISA kit will not cross-react with CA16 and can be used by the governmental agency to verify the exact contents of EV71 virus in the final product once it is developed. It is the only EV71-specific ELISA kit commercially available. As matter of fact, we have developed a CA16-specific monoclonal antibodies and are working on a CA16-specific sandwich ELISA (to be reported elsewhere).

Even though this ELISA kit has a LOD of 0.82 U/ml (1.6 ng/ml of total viral protein), it is not sensitive enough to detect the EV71 virus in human clinical samples. To develop it into a diagnostic kit, we have to improve the LOD to less than 0.2 ng/ml (equivalent to 100 $TCID_{50}$/ml). It has been proven to be very difficult with the current format of assay.

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