



Development of an efficient neutralization assay for Coxsackievirus A10

Dongxiao Liu¹ · Longfa Xu¹ · Rui Zhu¹ · Zhichao Yin¹ · Yu Lin¹ · Wangheng Hou¹ · Shuxuan Li¹ · Shuizhen He² · Tong Cheng¹ · Ningshao Xia¹

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Abstract

Coxsackievirus A10 (CVA10) recently has become one of the major pathogens of hand, foot, and mouth disease (HFMD) in children worldwide, but no cure or vaccine against CVA10 is available yet. Serological evaluation of herd immunity to CVA10 will promote the development of vaccine. The traditional neutralization assay based on inhibition of cytopathic effect (Nt-CPE) is a common method for measuring neutralizing antibody titer against CVA10, which is time-consuming and labor-intensive. In this study, an efficient neutralization test based on a monoclonal antibody (mAb) 3D1 against CVA10, called Elispot-based neutralization test (Nt-Elispot), was developed. In the Nt-Elispot, the mAb 3D1 labeled with horseradish peroxidase (HRP) was used to detect the CVA10-infected RD cells at a 1:4000 dilution and the optimal infectious dose of CVA10 was set at 10^5 TCID₅₀/well when combined with a fixed incubation time of 14 h. Compared with the Nt-CPE, the Nt-Elispot method effectively shortened the detection period and presented a good correlativity with it. Using the Nt-Elispot, a total of 123 sera from healthy children were tested for neutralizing antibody against CVA10, demonstrating that the overall seroprevalence was 49.3% (54/123) and the geometric mean titer (GMT) had been calculated as 574.2. Furthermore, 2 anti-CVA10 neutralizing mAbs were obtained by screening via the Nt-Elispot. Overall, the established Nt-Elispot could be used as an efficient and high-throughput method for evaluating immunity to CVA10 and screening the neutralizing antibodies.

Keywords Coxsackievirus A10 · Neutralizing antibody · Elispot · Serological epidemiology

Introduction

Coxsackievirus A10 (CVA10) belongs to the *Enterovirus genus* of the *Picornaviridae* family (Itagaki et al. 1987), which has recently emerged as one of the main causative agents of hand, foot, and mouth disease (HFMD) in children. Sporadic occurrences and outbreaks of HFMD cases related by CVA10 have increased in Asia (Koh et al. 2016; Xing et al. 2014; Yang et al. 2015), Africa (Gopalkrishna et al. 2012), and

Europe (Blomqvist et al. 2010; Bracho et al. 2011; Mirand et al. 2012) and resulted in a large burden of disease. In general, CVA10-associated HFMD is classically a mild disease, but some severe clinical manifestations such as onychomadesis (Bracho et al. 2011; Davia et al. 2011), convulsion (Chen et al. 2017), hyperCKemia (Okada et al. 2013), central nervous system disorders (Chen et al. 2017; Kumar et al. 2013), or even death were reported. Moreover, the co-infection and co-circulation of CVA10 with other enteroviruses can lead to more serious complications (Bracho et al. 2011). Vaccination has been considered the most effective strategy for HFMD control. Although some recent progress in developing vaccine (Lim et al. 2018; Shen et al. 2016) and therapy for CVA10 infection (Tan et al. 2018; Zhu et al. 2018b) have been reported, there is still no clinically available vaccine or cure accessible.

An efficient neutralization assay plays a major role in evaluating the efficacy of vaccines or antiviral reagents. The level of neutralizing antibody is regarded as a dominating indicator of protective immunity induced by vaccines or previous infection (Jin et al. 2016; Zhang et al. 2017; Zhu et al. 2018a). Traditionally, the standard neutralization test to detect

Dongxiao Liu and Longfa Xu contributed equally to this work.

✉ Tong Cheng
tcheng@xmu.edu.cn

¹ State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Life Sciences, School of Public Health, Xiamen University, Xiamen 361102, People's Republic of China

² Xiamen Center for Disease Control and Prevention, Xiamen 361012, People's Republic of China

neutralizing antibody levels against enteroviruses is the cytopathic effect (CPE)-based neutralization test (Nt-CPE) (WHO 1997). However, Nt-CPE has some shortcomings, such as long incubation periods and complex operations, and it is not suitable to apply to large sample tests. Therefore, an efficient and high-throughput neutralization assay needs to be developed.

The enzyme-linked immunospot (Elispot) assay has been verified as a highly sensitive, objective, and high-throughput immunological method (Ranieri et al. 2014). Each biomarker of interest produced in cell can sensitively be captured and detected by antibody, presenting as a spot via an enzyme-catalyzed color reaction. The number of antigen-specific cells is objectively determined by an Elispot analyzer, after automatically scanning and counting. In addition, this assay can be conducted in 96-well cell culture plates, which is greatly suitable for a high-throughput test. In recent years, several Elispot assays have been established for testing the level of neutralizing antibodies against Enterovirus 71 (EV71) (Mao et al. 2013), Coxsackievirus B3 (CVB3) (Yang et al. 2014), Coxsackievirus A16 (CVA16) (Hou et al. 2015), Echovirus (Echo25) (Li et al. 2016), EVD68 (Wan et al. 2017), Rotavirus (Li et al. 2014), and HSV-1 (Luo et al. 2016). However, this type of Elispot for CVA10 is not available yet. In this study, an efficient neutralization assay against CVA10, called Nt-Elispot, was successfully developed. A total of 123 sera from healthy children were tested for neutralizing antibody against CVA10 and 2 anti-CVA10 neutralizing mAbs were obtained by testing and screening using this assay.

Materials and methods

Cells, viruses, and serum samples

Rhabdomyosarcoma (RD) cells were obtained from the American Type Culture Collection (ATCC) and cultured in Minimal Essential Medium (MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) plus 100 µg/mL streptomycin, 100 U/mL of penicillin, and 2 mM L-glutamine. The CVA10 strain CVA10-FJ-01 (GenBank accession no. KY012321) was isolated in Fujian Province of China, which was propagated in RD cells and was maintained in State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics (Xiamen University, China). The CVA10 titer was expressed as the 50% tissue culture infectious doses (TCID₅₀), which was calculated by Reed-Muench method. The viruses were stored in aliquots at −80 °C. An anti-CVA10 neutralizing antibody-positive human serum sample, an anti-CVA10 neutralizing antibody-negative human serum sample, and a total of 123 serum samples from healthy children aged between 8 months and 6 years in Xiang'an district

of Xiamen City were provided by the Xiamen City Center for Disease Control and Prevention (XMCDC). The 123 serum samples were collected randomly in Xiang'an district in 2016. An anti-CVA10 neutralizing antibody-positive mice serum sample was collected from CVA10-immunized mice and an anti-CVA10 neutralizing antibody-negative mice serum sample were collected from non-immunized mice. All serum samples were stored at −20 °C and inactivated at 56 °C for 30 min before testing.

Production of mAbs

BALB/c mice were obtained from Slac Laboratory Animal Co., Shanghai, China. Five 6-week-old female BALB/c mice were immunized subcutaneously with the CVA10-FJ-01 strain of CVA10, which was emulsified in an equal volume of Freund's adjuvant (Sigma-Aldrich), and boosted twice at 2-week intervals. After the final boost, Sp2/0 myeloma cells were fused with spleen cells from immunized mice. The neutralization assay against CVA10 was applied for detecting all hybridoma supernatants. Antibodies were purified from the mouse ascitic fluid by protein A chromatography (GE Healthcare). All of the mAbs were conjugated to horseradish peroxidase (HRP) by the method that was reported by Nakane and Kawaoi (1974) and stored at −20 °C.

Binding ELISA

The supernatant of CVA10 cultures (1:5 diluted) was adsorbed at 100 µL/well to 96-well plates at 4 °C overnight, then every well was blocked by 200 µL skim milk for 2 h at 37 °C after the wash with PBST (0.05% Tween-20 in PBS). Various concentrations of mAb 3D1 (the initial concentration of 1 mg/mL, diluted five-fold serially) were added and incubated at 37 °C for 1 h. After five times washing with PBST, GAM-HRP was added into each well as the secondary antibody at 1:5000 dilutions and incubated at 37 °C for 30 min. Following five times washing with PBST, the plate was treated with TMB chromogen substrate for color development and was stopped by 2 M H₂SO₄, absorbance was measured at A450/620. The average OD450 readings from triplicate wells at each dilution were read.

Immunofluorescence assay

RD cells were pre-seeded on round glass coverslips in 24-well tissue culture plates and then infected with CVA10. After 12 h, cells were fixed with 4% paraformaldehyde for 30 min in the dark. Then cells were permeabilized with 0.2% Triton X-100 diluted in PBS for 30 min at room temperature and blocked with 5% goat serum for 1 h at 37 °C. After washing with PBS,

cells were incubated with mAb 3D1 (the initial concentration of 1 mg/mL at a dilution of 1:1000 in PBS) for 1 h at 37 °C. After three times washing with PBS, cells were stained with fluorescein isothiocyanate–conjugated goat anti-mouse antibody (GAM-FITC, at a dilution of 1:500 in PBS) for 30 min at 37 °C. After nucleus staining with 4',6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature, cells were observed using a fluorescent microscope (BX51, Olympus).

Nt-CPE

RD cells were seeded at 5×10^3 cells per well into 96-well plates more than 6 h prior to the experiment. Serum samples were serially diluted two-fold with MEM and the first dilution was 16-fold. Each diluted serum was incubated in 1:1 volume ratio with CVA10 (100 TCID₅₀) at 37 °C for 1 h and then added into pre-seeded cells. After incubation at 37 °C for 5–7 days, the CPE was observed by microscopy. All serum samples were tested in duplicate. The neutralizing titers were read as the highest dilution that completely inhibited CPE > 50% of the wells.

Nt-Elispot

RD cells were seeded at 2×10^4 cells per well into 96-well plates more than 6 h prior to the experiment. Serum samples or mAbs were serially diluted two-fold and incubated in 1:1 volume ratio with CVA10 (10⁵ TCID₅₀) at 37 °C for 1 h. The mixtures of samples and viruses were added into pre-seeded cells and then incubated at 37 °C for 14 h. All samples were tested in duplicate. For each plate, the cell control wells and the virus control wells should be set. After incubation, cells were detected by the Nt-Elispot assay as previously described (Yang et al. 2014). HRP-conjugated mAb 3D1 against CVA10 worked as the detection antibody at a 1:4000 dilution. After staining, the plates were scanned and the blue colored cells were counted by an ImmunoSpot Image analyzer 5.0 (Cellular Technology Ltd., USA). The inhibition rate of the sample was calculated using the following equation: $P = [1 - (N_{\text{test}} - N_{\text{cell control}}) / (N_{\text{virus control}} - N_{\text{cell control}})] \times 100\%$. In this equation, P is the inhibition rate of the sample, and N_{test} , $N_{\text{cell control}}$, and $N_{\text{virus control}}$ are the average number of spots in the test wells, cell control wells, and virus control wells respectively. The neutralization titer was defined as the highest dilution that completely inhibits > 50% of the spots.

Statistical analysis

The neutralizing titer, seroprevalence, and geometric mean titer (GMT) were calculated. All statistical analyses were performed using GraphPad Prism version 7.0.

Result

Characterizations of the monoclonal antibody 3D1

A panel of mAbs against CVA10 were acquired via the mice immunized with the supernatant of CVA10 cultures. A representative mAb 3D1, which exhibited high binding efficacy in ELISA (Fig. 1a), was selected as the detection antibody for the Nt-Elispot assay. The immunofluorescence assay showed that 3D1 had positive reactivity with CVA10-infected RD cells, but not with other enteroviruses species A such as EV71-, CVA16-, or CVA6-infected cells, and did not react with uninfected cells (Fig. 1b). Similarly, applied for the Nt-Elispot assay, the HRP-conjugated 3D1 (1 mg/mL) could only label the cells infected with CVA10 in blue, with the dilution of 1:4000 (Fig. 1b). These results indicated that mAb 3D1 was specific for CVA10.

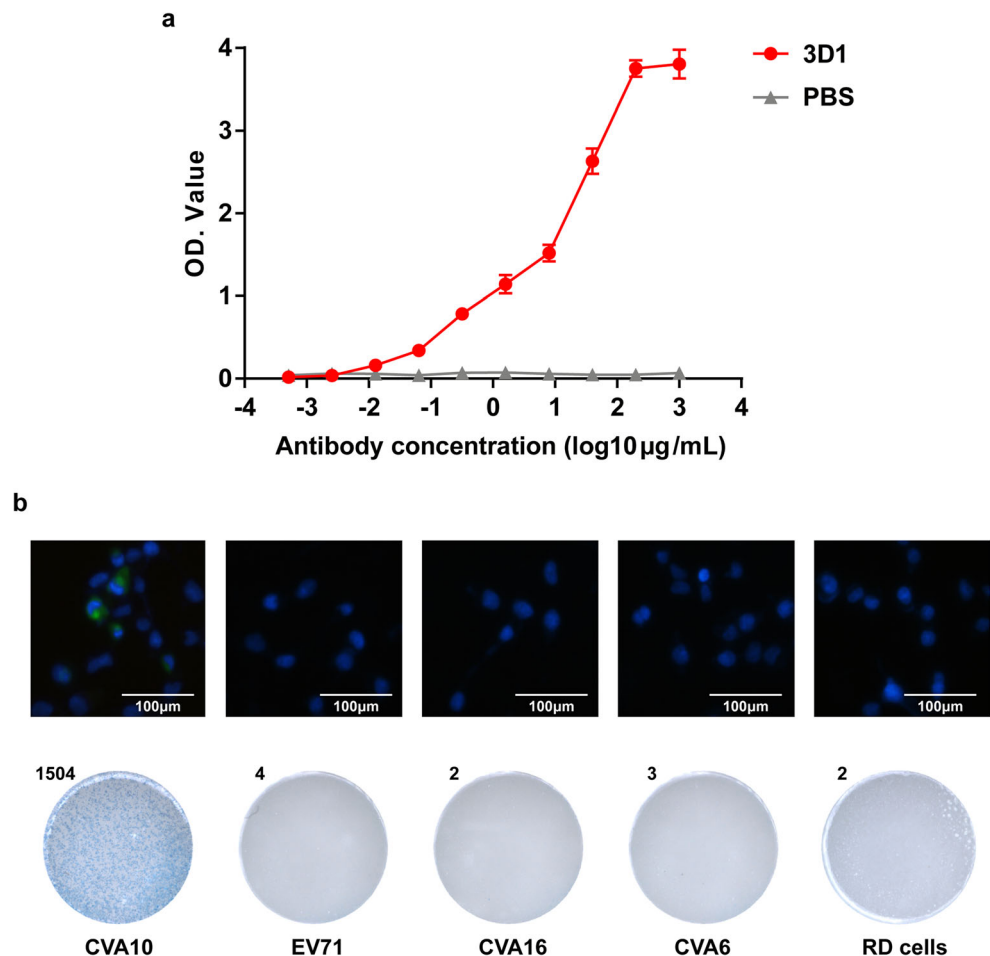
Determination of the optimal infectious dose for the Nt-Elispot

The infection kinetic of CVA10, which was influenced by infectious dose and incubation time, should be determined for the Nt-Elispot. To shorten the testing time, RD cells were incubated with different doses of CVA10, ranging from 10³ to 10⁶ TCID₅₀/well at 37 °C for 14 h. As shown in Fig. 2, the spots of the CVA10 group went up with the increasing of the infectious dose and peaked in the dose of 10⁵ TCID₅₀/well with about 1600 spots, and then began to decrease at higher doses of virus. The background was less than 100 spots in the dose of 10⁵ TCID₅₀/well in the inactivated CVA10 group and remained below 10 spots in the PBS group (Fig. 2). Thus, for the CVA10 Nt-Elispot, the optimal infectious dose was set at 10⁵ TCID₅₀/well when combined with a fixed incubation time of 14 h.

Comparison of the Nt-CPE and the Nt-Elispot

The traditional Nt-CPE is regarded as the standard method for detecting the neutralizing antibody level against enteroviruses and as a reference for evaluating other neutralization tests. In order to analyze the consistency between the Nt-CPE and Nt-Elispot, four samples of sera, a neutralizing antibody-positive human serum sample, a neutralizing antibody-negative human serum sample, a neutralizing antibody-positive mice serum sample, and a neutralizing antibody-negative mice serum sample were assayed. The neutralizing titers of these four samples were 2048, < 16, 8192, and < 16, respectively, as determined by the Nt-CPE. Then, these four serum samples were diluted serially in two-fold dilutions, tested by Nt-Elispot. The results showed that the neutralizing titers of both neutralizing antibody-positive

Fig. 1 Characterizations of the mAb 3D1. **a** Binding efficacy of 3D1 to CVA10 in ELISA. 96-Well plates were coated with CVA10 virus and diluted serially 3D1 were added. The amount of bound 3D1 was detected by HRP assay. The average OD450 readings from triplicate wells were displayed. **b** Specificity of the detection antibody 3D1. The results of immunofluorescence assay of CVA10-, EV71-, CVA16-, and CVA6-infected RD cells were shown in the upper line. The uninfected RD cells were used as negative control. The second antibody was GAM-FITC (green) and the nuclei were counterstained with DAPI (blue). The results of Nt-Elispot assay were displayed in the lower line. The CVA10-infected RD cells were labeled in blue with HRP-conjugated 3D1

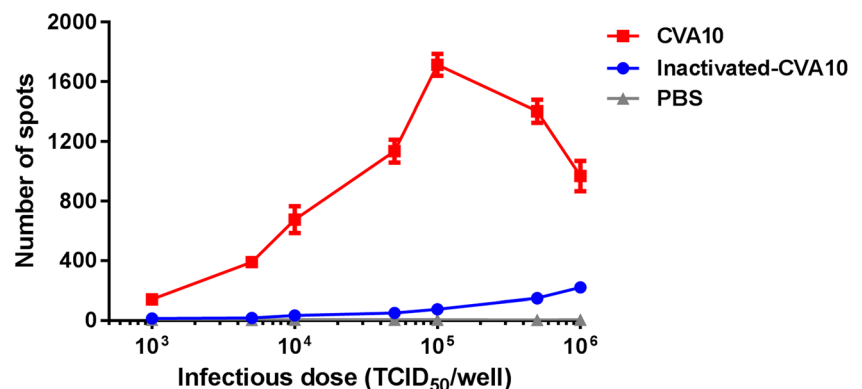


sera were decreased with the increasing dilution at a rate of two times, and the measured neutralizing titers had a good correlation with the predicted neutralizing titers (Fig. 3a, c). In addition, as expected, the neutralizing titer of negative sera was < 16, tested by Nt-Elispot (Fig. 3b, d). Therefore, these data indicated that the results of Nt-Elispot were consistent with those of Nt-CPE, which indicated Nt-Elispot presented a good correlativity with Nt-CPE.

Detection of serum neutralizing antibodies against CVA10 using Nt-Elispot

The infants and young children are susceptible to CVA10 infection. Using the Nt-Elispot, a total of 123 sera from healthy children aged between 8 months and 6 years were tested for neutralizing antibody against CVA10. A neutralizing titer of ≥ 16 was defined as a threshold value for positivity

Fig. 2 Determination of the optimal infectious dose for the Nt-Elispot. RD cells were incubated with different doses of CVA10, ranging from 10^3 to 10^6 TCID₅₀/well. Inactivated-CVA10 (blue line) was heat-inactivated at 56 °C for 30 min. Both the inactivated-CVA10 and CVA10 were incubated at 37 °C for 14 h. Each of dilution well was performed in triplicate and followed by detection for the Nt-Elispot



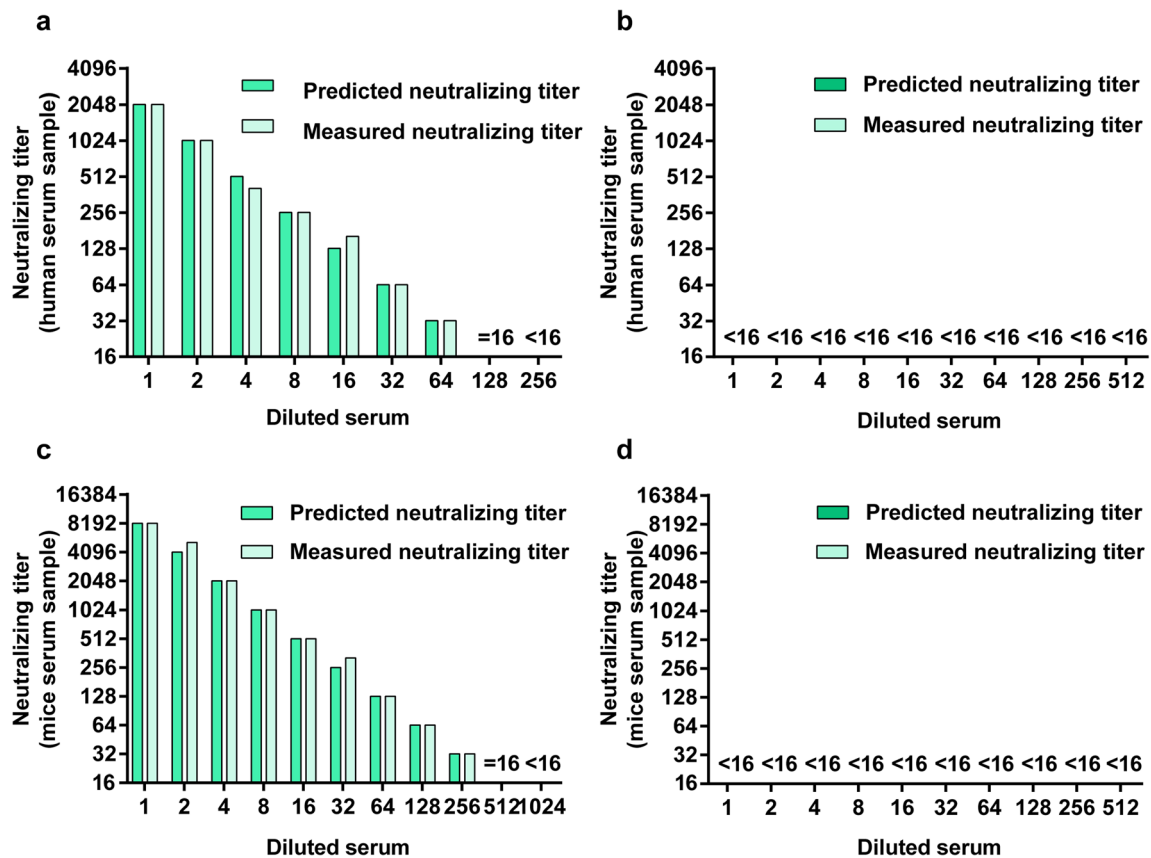


Fig. 3 Comparison of the Nt-CPE and the Nt-Elispot. **a** A neutralizing antibody-positive human serum sample, **b** a neutralizing antibody-negative human serum sample, **c** a neutralizing antibody-positive mice serum sample, and **d** a neutralizing antibody-negative mice serum sample were assayed. The predicted neutralizing titers of these four samples were

2048, < 16, 8192, and < 16, respectively, as determined by the Nt-CPE. The serum samples were serially diluted in two-fold dilutions and analyzed by Nt-Elispot. The results of Nt-Elispot were consistent with those of Nt-CPE

(Liu et al. 2011; Zhu et al. 2018a). As shown in Fig. 4a, the overall seroprevalence of CVA10 was 49.3% (54/123) and the geometric mean titer (GMT) has been calculated as 574.2. Furthermore, the seroprevalence was increased with age, ranging from approximately 20 to 70% (Fig. 4b), and the GMT was also increased with age and peaked in the age group of 3 to 4 years, with a neutralizing antibody titer of 301.9 (Fig. 4b). The results suggested that a high percentage of children had been exposed to CVA10 in Xiang'an district, especially those aged 3 to 4 years.

Screening of anti-CVA10 neutralizing antibody using Nt-Elispot

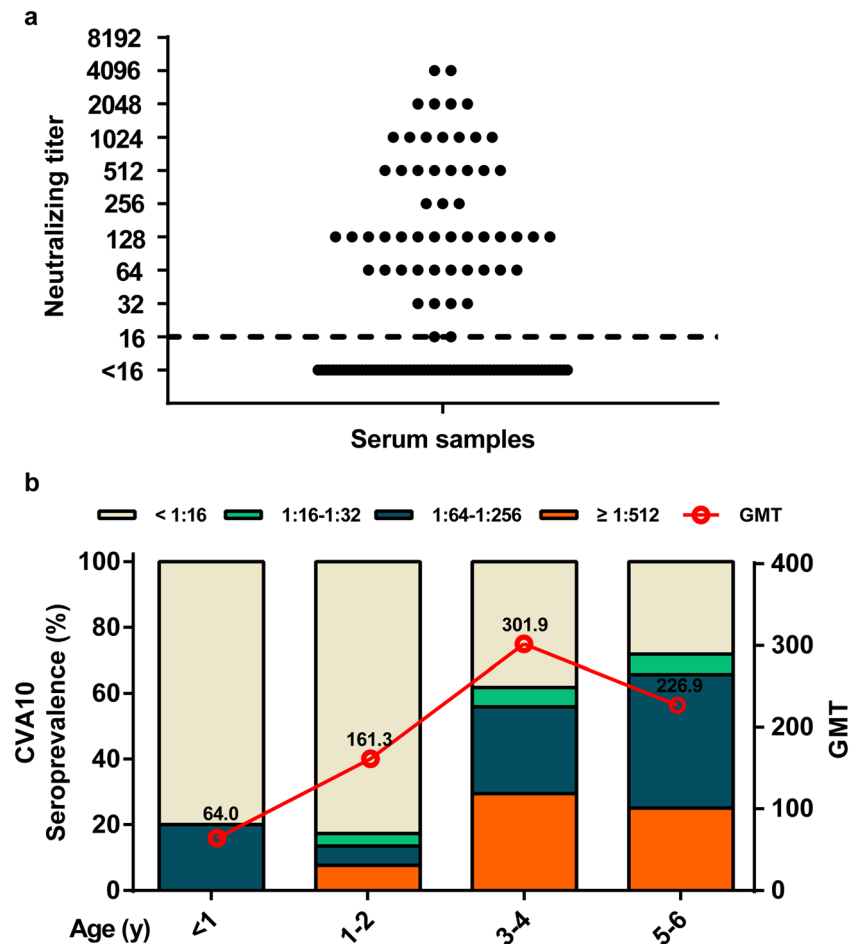
To gain the anti-CVA10 neutralizing antibodies, BALB/c mice were immunized with CVA10 and the splenocytes from the immunized mice were fused with sp2/0. Then, each 50 μ L hybridoma supernatant was screened by Nt-Elispot against CVA10. After three rounds of screening and cloning, 2 anti-CVA10 neutralizing mAbs were obtained. As showed in Fig. 5a, mAb 7B4 and mAb 8D6 can effectively neutralize CVA10 in vitro, while the control anti-CVA6 mAb 1D5 (Xu

et al. 2017) cannot. To determine the neutralizing titers of mAbs, 7B4 and 8D6 were diluted serially in two-fold dilutions (the initial concentration of 1 mg/mL), followed by Nt-Elispot. The results showed that the neutralizing titers of mAb 7B4 and 8D6 were 1024 and 512, respectively (Fig. 5b).

Discussion

HFMD is a common infectious disease that mainly affects children under 5 years of age, leading to considerable morbidity and mortality worldwide. EV71 and CVA16 are previously the predominant pathogens of HFMD; however, a large number of HFMD cases associated with CVA10 infection were reported in recent years (Koh et al. 2016; Xing et al. 2014; Yang et al. 2015). Humoral immunity typically plays a central role in the defense of enterovirus infection (Chung et al. 2008). The evaluation of the neutralizing antibody levels against CVA10 in population is essential for understanding the status of herd immunity and facilitating the development of vaccines and antiviral reagents. The CPE-based

Fig. 4 Detection of serum neutralizing antibodies against CVA10 using Nt-Elispot. **a** A total of 123 serum samples were collected from healthy population between 8 months and 6 years of age in Xiang'an district of Xiamen City. The neutralizing antibody titers against CVA10 were detected by Nt-Elispot. A neutralizing titer of ≥ 16 was defined as a threshold value for a positive score. **b** The Seroprevalence rate was illustrated via histogram. To show different levels of neutralizing antibody, the titers were divided into four ranges: $\geq 1:512$ (high), 1:64–1:256 (moderate), 1:16–1:32 (low), and $< 1:16$ (no). The geometric mean titer (GMT) was calculated as the point plot noted



neutralization assay remains the “gold standard” detection method for neutralizing antibody levels, but this traditional method is time-consuming and labor-intensive, which is not applicable to test a variety of samples. In this study, we developed a neutralization assay (Nt-Elispot) based on a specific

mAb 3D1 against CVA10. Compared to the Nt-CPE, the incubation time of this experiment is shortened from more than 3–5 days to 14 h. The Nt-Elispot quantifies the CVA10-infected cells based on the expression of viral protein, which can be bound by HRP-labeled mAb 3D1. After staining, an

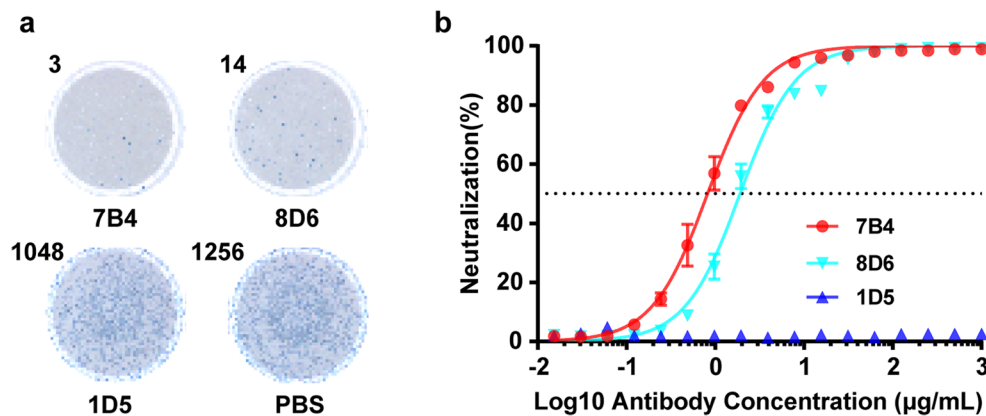


Fig. 5 Screening of anti-CVA10 neutralizing antibody using Nt-Elispot. **a** The neutralizing efficacy of mAbs, 7B4, and 8D6. Both of them were detected by modified Nt-Elispot. 1D5, an antibody of CVA6, and PBS were used as controls. The CVA10-infected cells were

labeled in blue with HRP-conjugated 3D1. **b** The neutralizing titers were calculated by Nt-Elispot as described in the material. Antibodies were diluted serially in two-fold dilutions (the initial concentration of 1 mg/mL)

Elispot analyzer can automatically scan and count the stained plaques without detection under a microscope. Therefore, the results are more stable and objective. Moreover, this method can be performed in 96-well cell culture plates, which is favorable for high-throughput detection. Previous studies demonstrated that there was a negative linear correlation between the infectious dose and the incubation time (Yang et al. 2014). For convenience and decrease in the testing time, the optimal CVA10 infectious dose and the incubation time were set at 10^5 TCID₅₀/well and 14 h, respectively.

Xiamen is a coastal tourist city in Fujian Province, which is divided into six districts, with approximately 27,000 cases of HFMD reported from 2008 to 2015 (He et al. 2017). Moreover, Chen et al. found that the proportion of CVA10 infections in severe HFMD cases in Xiamen City increased sharply from 0% in 2011, 2012, and 2014 to 38.7% in 2015 (Chen et al. 2017). Among six districts in Xiamen, the annual morbidity of overall HFMD cases in Xiang'an district ranked second, with a rate of 1400/1,000,000 (He et al. 2017). In our previous study, we conducted a serological survey of neutralizing antibodies to eight major enteroviruses among healthy people aged 5 months to 83 years, which found that the overall seroprevalence of CVA10 was 42.7% in Xiamen City and the seroprevalence in Xiang'an district was the highest with a rate of 57.3% (Zhu et al. 2018a). These results demonstrated that CVA10 may be one of the emerging agents of HFMD in Xiamen City, especially in Xiang'an district. The infants and young children are more susceptible to HFMD. To further characterize the seroepidemiology of CVA10 among children, in this study, we tested the neutralizing antibodies against CVA10 among 123 healthy children aged 8 months and 6 years in Xiang'an district by Nt-Elispot. The result showed that the overall seroprevalence of CVA10 among children was 49.3%, which further indicated that CVA10 was prevalent in the region.

In summary, the established CVA10 Nt-Elispot can be a sensitive, easy, and high-throughput method for anti-CVA10 neutralizing antibodies detection and served as a useful tool for CVA10 vaccine evaluation and serology surveillance. Furthermore, positive hybridoma supernatant against CVA10 could be objectively screened by Nt-Elispot.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animal experiments were approved by Xiamen University Laboratory Animal Center (XMULAC) and conducted in accordance with animal ethics guidelines and approved protocols. The Animal Ethics Committee approval number was XMULAC20160049. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committees of the Xiamen City Center for Disease Control and Prevention and the Xiamen University.

Informed consent Informed consent was obtained from all individual participants included in the study.

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