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Protein microarray-mediated detection of antienterovirus antibodies in serum

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Abstract

Objective: To utilize prokaryotic gene expression and protein microarray to develop and evaluate a sensitive, accurate protein microarray assay for detecting antienterovirus antibodies in serum samples from patients with hand, foot and mouth disease (HFMD). Enterovirus 71 (EV71) and coxsackievirus A16 (CA16), two common causative agents for HFMD, were used for assay development.

Methods: Serum was collected from patients with HFMD and healthy controls. EV71 and CA16 VP1 and VP3 genes were expressed in transfected *Escherichia coli*; the resultant VP1 and 3 proteins were used in a microarray assay for human serum EV71 and CA16 immunoglobulin (Ig) M and IgG. To validate the microarray assay, serum samples were tested for EV71 IgM using enzyme-linked immunosorbent assay (ELISA).

Results: Out of 50 patients with HFMD, EV71 IgM and CA16 IgM was detected in 80% and 44% of serum samples, respectively, using protein microarray, and EV71 IgM was detected in 78% of samples using ELISA. Protein microarray and ELISA showed 100% specificity for EV71-IgM detection.

Conclusion: The protein microarray assay developed in the present study shows potential as a sensitive technique for detecting EV71 IgM in serum samples from patients with HFMD.

Keywords

Hand, foot and mouth disease, EV71, CA16, protein microarray

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Introduction

Enterovirus 71 (EV71) and coxsackievirus A16 (CA16) are two of the most common causative agents of hand, foot and mouth disease (HFMD).[1] EV71 may cause various neurological diseases such as aseptic meningitis, acute flaccid paralysis and fatal encephalitis;[2] several EV71 outbreaks, associated with severe neurological complications, have been reported in Western Pacific countries or regions.[3–6] HFMD has emerged as the most prevalent infectious disease among children in China. The pathogenesis underlying EV71 and CA16 infections remains unclear and although no vaccines or antiviral therapies are available for the prevention or treatment of EV71 infection, progress has been made in recent years. Several compounds with a variety of mechanisms of action have been shown to inhibit EV71 replication, but none has been advanced to human clinical trials.[7,8] An effective approach to prevent EV71 outbreaks would be to develop a vaccine with a favourable safety and efficacy profile, and EV71 vaccine development has been underway in China and Singapore, with three companies in China completing Phase III clinical trials of inactivated EV71 vaccines.[9,10] Thus, determination of the pathogen causing HFMD, for effective early treatment, is desirable.

Virus isolation using cell/tissue culture remains the gold standard for enterovirus diagnosis.[11] Molecular methods, such as polymerase chain reaction (PCR) techniques including reverse transcription (RT)-PCR and real-time PCR, have been developed to detect EV71 and CA16 viral RNA in patients with HFMD.[12–15] In addition, enzyme-linked immunosorbent assay (ELISA) techniques have been developed to detect antibodies for the clinical diagnosis of HFMD.[16,17] The aim of the present study was to develop a rapid, sensitive and more specific and effective diagnostic method for detecting EV71 in clinical specimens.

The genus *Enterovirus* belongs to the *Picornaviridae* family and consists of 66 different subtypes, including polioviruses, coxsackievirus group A and coxsackievirus group B, echoviruses, and enteroviruses.[18] The different genogroups of EV71 are widely distributed around the world.[2] The EV71 genome possesses ~7 500 nucleotides and encodes four structural capsid proteins (VP4, VP2, VP3 and VP1), and seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D). The structure protein VP1 is considered to be variable and to play an important role in characterizing antigenicity.[18,19] Few studies have investigated EV71 VP3 structure protein to characterize antigenicity, and data are limited.[20]

Another form of medical diagnosis uses detection of particular antibodies. Antibodies exist as different isotypes, namely immunoglobulin (Ig)A, IgD, IgE, IgG and IgM. IgM antibodies appear early in the course of infection, then disappear (IgM titres usually fall within 2 months and normalize within 4–6 months); IgM usually reappears to a lesser extent following further antigen exposure. After an acute infection, elevated IgG levels may persist for several years and occasionally be detectable over the 3 years following acute infection.[21,22]

In the present study, VP1 and VP3 were selected as antigens to detect antibodies (IgG and IgM) in serum samples from patients with HFMD using protein microarrays, with the aim of developing this assay technique as a convenient, sensitive and economic diagnostic tool for HFMD.

Patients and methods

Study population and serum samples

This study sequentially recruited patients with acute HFMD who were admitted to Beijing YouAn Hospital, Beijing, China between February 2012 and February 2014. Inclusion criteria were as follows: children aged <5 years; clinical features of

HFMD (including fever, sore throat, ulcers in the throat, mouth and tongue, headache, and rash with vesicles on the hands, feet and inguinal area); presence of EV71 and (or) CA16 RNA in vesicular fluid, blood and urine samples, detected using RT-PCR in the clinical laboratory of Beijing YouAn Hospital. Data from the prior RT-PCR analyses were compared with the protein microarray results from the present study, to evaluate the microarray assay.

Serum samples were collected from patients enrolled in the study as follows: using standard methods, 2 ml of venous blood was collected between day 1 and day 5 following onset of fever or skin lesions. Blood samples were allowed to clot at 4°C for 24 h. Samples were then centrifuged at 3000–4000 r.p.m. at 4°C for 2 min. The serum from each sample was transferred to a new tube and stored at 4°C prior to use. Serum samples were also collected from healthy blood donors living in Beijing. All blood from donations was routinely screened for blood-borne pathogens, including: HIV, hepatitis B virus, hepatitis C virus, syphilis, malaria and bacterial contamination, using standard screening techniques.

The study protocol was approved by the Ethics Committee of Beijing YouAn Hospital, Beijing, China. Written informed consent was obtained from the patients or their legal proxies. Verbal informed consent was obtained from the healthy controls.

Amplification and cloning of viral VP1 and VP3 genes

Full-length EV71 and CA16 cDNAs (provided as gifts from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China), were used as templates for amplification of the peptide coding region. EV71 and CA16 VP1 and VP3 protein sequences were retrieved from the National Center for Biotechnology Information (NCBI) Entrez Protein Database. PCR amplification of four genes (EV71 VP1, EV71 VP3, CA16 VP1 and CA16 VP3) was performed using ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma-Aldrich, St Louis, MO, USA) and the following primer sequences:

EV71 VP1 forward, 5′-GCGGATCCGG AGATAGGGTGGCAGAT-3′;
EV71 VP1 reverse, 5′-GCGAATTCAAGA GTGGTGATCGATGT-3′;
EV71 VP3 forward, 5′-GCGAATTCGGG TTCCCCACCGAGCTGA-3′;
EV71 VP3 reverse, 5′-GCCTCGAGCTGG ATGGTGCCCGTCTG-3′;
CA16 VP1 forward, 5′-GCGCGGCCGCG GGGATCCTATTGCAGAT-3′;
CA16 VP1 reverse, 5′-GCCTCGAGTTAA TGGTGATGGTGATGGTG-3′;
CA16 VP3 forward, 5′-GCGCGGCCGC GGCATACCAACAGAGCTC-3′;
CA16 VP3 reverse, 5′-GCCTCGAGTTG TATATTAGCCGTTTG-3′.

Each 50-µl reaction contained 1 µl of forward primer, 1 µl of reverse primer, 5 µl template DNA, 25 µl of 2 × ReadyMix™ Taq PCR Reaction Mix, and 18 µl H_2O. The cycling programme involved preliminary denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 60 s, and elongation at 72°C for 60 s, followed by a final elongation step at 72°C for 20 min. The resultant four PCR products were each cloned into pET-28a expression vectors (EMD Millipore, Billerica, MA, USA) for production of His-tagged fusion proteins. The pET-28a vector and amplified gene were digested with the following restriction enzymes (New England Biolabs, Beverly, MA, USA), respectively for 1 h at 37°C according to the manufacturer's instructions: EcoR1 and XhoI (EV71 VP3); BamHI and EcoRI (EV71 VP1); SmaI and XhoI (CA16 VP3 and CA16 VP1). Plasmid DNA and PCR fragments were then purified using

QIAprep® Spin Miniprep Kit and QIAquick® Gel Extraction and PCR purification kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. T4 DNA ligase enzyme (Fermentas, St. Leon-Rot, Germany) was used to insert the digested and purified PCR fragment into the digested vector (incubated for 1 h at 22°C).

Cloning was confirmed by the release of insert from the vector on double digestion with the restriction enzymes EcoR1 and XhoI (EV71 VP3) in NEBuffer 3.1 (New Engand Biolabs) at 37°C for 2 h, BamHI and EcoRI (EV71 VP1) in NEBuffer 3.1 at 37°C for 2 h, and Sma I and Xho I (CA16 VP3, CA16 VP1) in CutSmart® Buffer first at 25°C with SmaI for 2 h, then with XhoI added, raised to 37°C for 2 h. The expression plasmid was then sequenced by Sino Geno Max Co., Ltd, Beijing, China using a genetic analyser (ABI Prism® 3700, Applied Biosystems Carlsbad, CA, USA).

Expression and purification of VP1 and VP3 recombinant proteins

Escherichia coli M15 (pREP4) cells (Qiagen, Hong Kong, China) were transfected with each of the four pET-28a enterovirus constructs to obtain expression of CA16 and EV71 His-tagged proteins. Next, 2 µl of recombined plasmid DNA was added to 15 µl pREP4 cells, Following 30 min incubation period on ice, a mixture of pREP4 and viral DNA was placed at 42°C for 45 s (heat shock), then placed back on ice. SOC media (20 g/l tryptone, 5 g/l yeast extract, 4.8 g/l $MgSO_4$, 3.603 g/l dextrose, 0.5 g/l NaCl, 0.186 g/l KCl) was added and the transformed cells were incubated at 37°C for 30 min with agitation. For production of recombinant fusion protein, the inserted clones were incubated in Luria broth base medium with added ampicillin (50 µg/ml) at 37°C to reach a density of 0.5 in OD_{600}, then protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside. Following culture of transfected *E. coli*, His-tagged recombinant proteins were purified using Ni-NTA resin (Genscript, Hong Kong, China). The proteins were eluted with 6 M guanidine hydrochloride buffer (pH 7.9), then precipitated with trichloroacetic acid. Fractions containing the protein were identified by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE). The proteins were renatured by serial dialysis into 1×phosphate-buffered saline (PBS)-0.25 M arginine buffer (pH 8.0), and protein purity was analysed by SDS–PAGE. Concentrations of the purified viral proteins were determined using a Beckman Coulter DU730 spectrophotometer (Beckman Coulter Commercial Enterprise (China) Co., Ltd. Shanghai, China).

Protein microarray

The purified EV71 VP1, EV71 VP3, CA16 VP1 and CA16 VP3 viral proteins in 1×PBS-0.25 M arginine buffer, with 30% glycerol (to prevent evaporation of nanodroplets) were spotted in quadruplicate, at 1 mg/ml, onto aldehyde group-modified glass slides. Following spotting, slides were incubated for 24 h at 4°C (to facilitate complete protein immobilization), then immobilized. Slides were then blocked with 5% bovine serum antigen (BSA) for 2 h at 37°C, then washed three times with 1×PBS (pH 7.4) for 30 s each wash. Serum samples (1 µl) from patients with HFMD and controls were diluted 20×in blocking buffer (0.5% BSA in PBS containing 0.1% Tween-20, pH 7.4) and hybridized for 30 min at 37°C. Samples were then washed twice with PBS-Tween 20 (PBST) with a final rinse using PBS. For detecting IgG antibody, 10µl of cyanine (Cy)3-labelled goat antihuman IgG (1 : 1000, KPL, Gaithersburg, MD, USA) was added. For detecting IgM antibody, 10µl of

CyTM3-labelled goat antihuman IgM (1 : 500, KPL) was added. Slides were then incubated for 30 min at 37°C in the dark and then rinsed twice with washing buffer (PBS-0.1% Tween-20 [PBST]) and once with H_2O.

Slides were scanned using a GenePix® 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA) in the fluorescence acquisition mode. For Cy3-labelled probes, a 532 nm excitation laser source was chosen, while the photomultiplier tube voltage was set at 500 volts. For scan resolution, the pixel size was set as 10 μm. The image was initially analysed using GenePix® Pro microarray acquisition and analysis software, version 4.0.1.23 in Array Analysis mode for absolute fluorescence intensity measurement. For each slide, a reference channel image was used to set a grid for locating an individual spot; fluorescence readings for each spot were recorded. Raw data were exported into Microsoft Excel® for storage and further analysis.

Detection of IgM anti-EV71 VP1 by ELISA

To evaluate the performance of the protein microarray assay, all serum samples from patients with HFMD were analysed using Wantai EV-71 IgM ELISA kits (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, China) according to the manufacturer's instructions. To evaluate the performance of the Wantai EV-71 IgM ELISA kits, a random selection of serum samples from patients with HFMD were analysed using in-house ELISA assays as follows: anti-EV71 VP1 IgM was detected using an IgM μ-chain capture ELISA. First, 96-microwell immunoassay strips (Nunc, USA) were coated with goat antihuman IgM (μ chain- specific, Jackson, USA) diluted in a carbonate buffer (pH 9.6). The strip was incubated at 4°C overnight, then washed four times with PBST. Plates were then blocked with 250 μl of blocking buffer (PBS, 5% nonfat dry milk, 0.5% BSA (pH 7.4) at room temperature for 4 h before being washed four times with PBST. Then, 50μl of a 10 × dilution of the serum specimen, blank (dilution buffer; pH 7.4), or positive (HFMD sample) and negative (healthy control sample) were each added to separate wells, and the strips were incubated at 37°C for 1 h. After washing twice with PBST and final rinse with PBS, 50 μl of purified EV71 VP1-biotin (1 mg/ml) was added to each well, and samples were again incubated at 37°C for 1 h. Then, 100μl of a 1000×dilution of horseradish peroxidase (HRP)-conjugated streptavidin (Chemicon, CA, USA) was added to each well, the samples were incubated at 37°C for 1 h and washed 5 times with PBST, then 3,3′,5,5′-Tetramethylbenzidine (TMB)/E substrate (Chemi-Con, Buena Park, CA, USA) (100 μl) was added to each well. The strip was incubated in the dark at room temperature for 30 min, and the reaction was terminated by the addition of 100μl 2 N H_2SO_4. Optical density (OD) was read at 450 nm with a reference filter set to 620 nm using a Thermo Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analyses

Statistical analyses were performed using χ^2-test with ZIYUE software, version 1.61 (http://down.tech.sina.com.cn/content/29444.html). A P-value < 0.05 was considered to be statistically significant.

Results

Preparation of antigenic viral recombinant proteins

Viral VP1 and VP3 genes from CA16 and EV71 enteroviruses were cloned into pET-28a (+) expression vectors. All four constructs were confirmed by DNA sequencing and expressed in *E. coli* M15 (pREP4). VP1

and VP3 protein products were purified and analysed as described above. All of the expressed proteins migrated between the 25 and 37 kDa markers, which was consistent with the predicted size of each protein (Figure 1a). All four recombinant proteins were used to generate the protein microarray.

Protein microarray

Serum samples from patients with HFMD. A total of 50 serum samples were collected from patients with HFMD and tested using the protein microarray. A signal log ratio value of 0 was chosen as the cut-off value to distinguish between positive and negative test results. A signal log ratio ≥ 0 was indicative of a positive sample and a ratio <0 was indicative of a negative sample. The results of signal log ratio analyses were consistent with unaided observations using the naked eye. The protein microarrays were analysed following incubation of serum from healthy control subjects (Figure 1b) and from patients with HFMD (Figure 1c). Anti-EV71VP1 IgM immunoreactivity was detected in 80% of samples from patients with HFMD, and was higher than anti-EV71VP3 IgM, anti-CA16VP1 IgM and anti-CA16VP3 IgM immunoreactivity (observed in 40%, 44%, and 20% of samples, respectively; Table 1). The results of protein microarray analysis of serum samples were consistent with the RT–PCR results performed by the clinical laboratory prior to enrollment in the present study: The total positive rate of prior RT–PCR for EV71 and CA16 were 76% (38/50) and 24% (12/50), respectively: 35 samples were positive for EV71 alone and nine samples were positive for CA16 alone; three samples were positive for both EV71 and CA16; three samples were negative for both EV71 and CA16.

Anti-EV71 IgG and CA16 IgG antibodies were also detected using the protein microarray. Anti-EV71VP1 IgG, anti-EV71VP3 IgG, anti-CA16VP1 IgG, and anti-CA16VP3 IgG immunoreactivity were detected in 70%, 18%, 72%, and 54% of serum samples, respectively, from 50

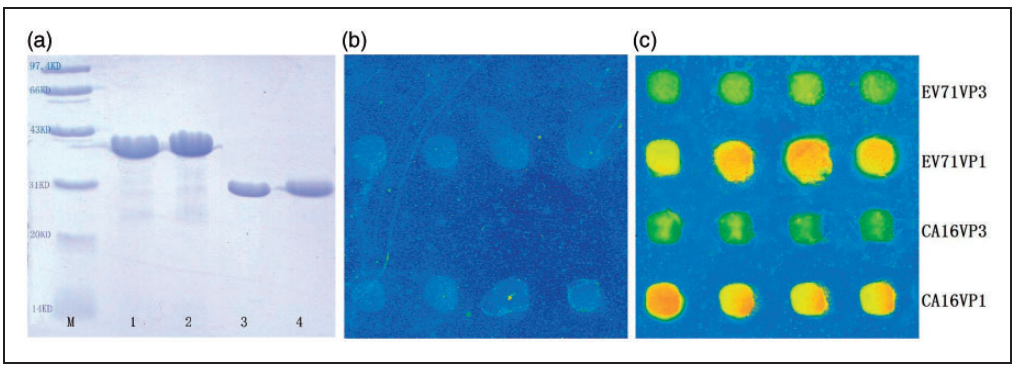

Figure 1. (a) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis image of four purified His-tagged recombinant viral proteins. Enterovirus (EV)71 VP1 (lane 1), coxsackievirus (CA)16 VP1 (lane 2), EV71 VP3 (lane 3), and CA16 VP3 (lane 4), and molecular weight ladder (lane M). Representative GenePix® 4000B microarray fluorescence scan image of protein microarray analysis to detect IgM antibodies against EV71 VP3, EV71 VP1, CA16 VP3 and CA16 VP1 proteins in human serum from (b) a healthy control subject showing negative antibody results and (c) a patient with hand, foot and mouth disease showing positive antibody results. The colour version of this figure is available at: http://imr.sagepub.com

Table 1. Protein microarray and enzyme-linked immunosorbent assay (ELISA) analysis the presence of antibodies against the enterovirus proteins enterovirus (EV)71 VP1, EV71 VP3, coxsackievirus (CA)16 VP1, and CA16 VP3 in serum samples from patients with hand, foot and mouth disease (HFMD) and healthy control subjects.

Assay	Antibody		Patients with HFMD	Positive result	Healthy controls	Positive result
Protein microarray	IgG	EV71VP1	50	35 (70.0)	30	9 (30)
		EV71VP3	50	9 (18.0)	30	2 (6.7)
		CA16VP1	50	36 (72.0)	30	18 (60.0)
		CA16VP3	50	27 (54.0)	30	1 (3.3)
	IgM	EV71VP1	50	40 (80.0)	30	0
		EV71VP3	50	20 (40.0)	30	0
		CA16VP1	50	22 (44.0)	30	0
		CA16VP3	50	10 (20.0)	30	0
IgM μ-chain capture ELISA	IgM	EV71	20	13 (65)	20	0
Wantai EV71-IgM ELISA kit	IgM	EV71	50	39 (78.0)	30	1 (3.3)

Data presented as *n* (%) of samples.
Ig, immunoglobulin.
There were no statistically significant differences between the ELISA and protein assays in either group ($P > 0.05$; χ^2-test).

patients with HFMD (Table 1). The data also showed that any sample with immunoreactivity for EV71VP3-IgM, CA16VP1-IgM and CA16VP3-IgM was also positive for EV71VP1 IgM.

Serum samples from healthy controls. Serum samples obtained from 30 healthy blood donors were tested in parallel to evaluate the specificity of the protein microarray. Anti-EV71VP1 IgG was detected in nine of 30 samples (30%) and anti-CA16VP1 IgG was detected in 18/30 samples (60%, Table 1). Only two samples (6.7%) were positive for EV71VP3 IgG and one (3.3%) was positive for CA16VP3 IgG. False-positive reactions for IgM were not found in healthy serum samples (Table 1).

IgM capture ELISA results

A total of 20 serum samples from patients with HFMD were analysed using the in-house ELISA. An absorbance value 0.2 was chosen as the cut-off value, thus, an ELISA value ≥ 0.2 was defined as positive. Anti-EV71VP1 IgM was detected in 13/20 serum samples (65%) from patients with HFMD and two of the samples were negative (Table 1).

Thirty-nine of the 50 serum samples from patients with HFMD (78%) were positive for EV-71 IgM (Table 1).

Discussion

Protein microarray technology is a powerful tool for high-throughput assays of protein expression, protein-protein interaction and enzyme activity,[23,24] and has become an effective way to diagnose many diseases because many disease-related proteins are detectable in serum.[25–27]

In the present study, antibodies in serum from patients with HFMD were detected using four kinds of recombinant antigenic probes specific for EV71VP1, EV71VP3, CA16VP1, and CA16VP3, which were

immobilized as capture probes at unique positions on a slide. The protein microarray was able to detect neutralizing antibodies against EV71 and CA16, and to detect IgM and IgG antibodies against EV71 and CA16 by hybridizing different secondary antibodies. Anti-EV71VP1 IgM was highly detectable in serum samples from patients with HFMD, and almost all anti-VP3-positive serum samples were also positive for antibodies against VP1 protein. In the present study, the detection pattern of serum antibodies by protein microarray using enterovirus VP1 and VP3 proteins as antigens suggested that the immunological reactions to VP1 and VP3 of EV71 and CA16 were different. IgM against VP1 but not VP3 appeared to be generated in patients with HMFD, which needs to be clarified further.

In the present study, a proportion of healthy control samples tested positive for anti-EV71 IgG and anti-CA16 IgG using the microarray chip, which may have been due to previous EV71 (and/or CA16) infection. Levels of IgM antibody increase continuously before peaking at the second week following symptom onset, then decline gradually.[28] During the acute phase (<7 days following symptom onset), EV71 IgM has been detected in 88% of samples tested using ELISA.[29] Serum from patients with HFMD and healthy controls could not be distinguished by detecting IgG using microarray in the present study, however, no commercial ELISA assay was available for detecting CA16-IgM, EV71-IgG and CA16-IgG. This meant that appropriate control methods for CA16-IgM, EV71-IgG and CA16-IgG were lacking in the present study.

In the present study, anti-EV71 (and/or CA16) IgM was useful for diagnosing acute HFMD. Some patients were positive for both EV71 and CA16, perhaps because patients with HFMD could be infected by both EV71 and CA16 concomitantly. Co-infections with different HFMD pathogens have been reported previously. In Shanghai, co-infection with EV71 and CA16 was observed in 17.6% of total CA16 cases between 2009 and 2010.[30]

In the present study, anti-EV71 and CA16 IgM antibodies were undetected in 10 of 50 samples with the EV71 assay, perhaps because the antibody titres were too low for detection at the onset of the infection.

Studies show that CA16 infected patients and EV71 infected patients present high cross-neutralization antibodies against each other.[31,32] Alignment of two (EV71 and CA16) protein sequences using BLAST (bl2seq) online (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the protein sequence of EV71 was 77% homologous with that of CA16 (author's own data). In the present study, the antibody against serum EV71 was a polyclonal antibody which crossreacted with CA16 antigen. The present data also showed that any sample that tested positive for CA16 VP1- and CA16 VP3-IgM was also positive for EV71-VP1 IgM. Although there are many methods to detect anti-EV71 or anti-CA16 antibodies, it remains difficult to discriminate between anti-EV71 or anti-CA16 antibodies in serum.

The protein microarray method used in the present small study appeared to show excellent specificity, i.e. no false-positive results were detected in healthy controls. The present microarray results (with a positive rate for EV71-IgM of 80%) compared favourably with the gold standard ELISA method of 78% and suggest that the sensitivity of the protein microarray method is at least equal to, or perhaps slightly higher than the ELISA. Protein microarray is more economical than ELISA,[33] making its use potentially more attractive in developing countries. This small microarray may save resources such as antibodies, antigens and other experimental materials, and is a faster technique compared with ELISA (the protein microarray takes ~1.5 h to detect

multiple antibodies; ELISA takes about 3 h to detect only one antibody). Protein microarray technology, therefore, appears to allow rapid, easy and parallel detection of multiple elements in a single assay.

In conclusion, a protein microarray assay for detecting EV71-IgM in serum samples from patients with HFMD was developed in the present study. The protein microarray is a potential alternative method for detecting antibodies in serum samples, and as it is relatively easy to perform and low in cost, it may be suitable for clinical diagnosis and use in public health.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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References

1. Li L, He Y, Yang H, et al. Genetic characteristics of human enterovirus 71 and coxsackievirus A16 circulating from 1999 to 2004 in Shenzhen, People's Republic of China. *J Clin Microbiol* 2005; 43: 3835–3839.
2. Goto K, Sanefuji M, Kusuhara K, et al. Rhombencephalitis and coxsackievirus A16. *Emerg Infect Dis* 2009; 15: 1689–1691.
3. Wu JS, Zhao N, Pan H, et al. Patterns of polymorphism and divergence in the VP1 gene of enterovirus 71 circulating in the Asia-Pacific region between 1994 and 2013. *J Virol Methods* 2013; 193: 713–728.
4. Liu CC, Chou AH, Lien SP, et al. Identification and characterization of a cross-neutralization epitope of Enterovirus 71. *Vaccine* 2011; 29: 4362–4372.
5. Lin TY, Hsia SH, Huang YC, et al. Proinflammatory cytokine reactions in enterovirus 71 infections of the central nervous system. *Clin Infect Dis* 2003; 36: 269–274.
6. Zhang Y, Zhu Z, Yang W, et al. An emerging recombinant human enterovirus 71 responsible for the 2008 outbreak of hand foot and mouth disease in Fuyang city of China. *Virol J* 2010; 7: 94.
7. Cui S, Wang J, Fan T, et al. Crystal structure of human enterovirus 71 3C protease. *J Mol Biol* 2011; 408: 449–461.
8. Shang L, Xu M and Yin Z. Antiviral drug discovery for the treatment of enterovirus 71 infections. *Antiviral Res* 2013; 97: 183–194.
9. Liang ZL and Wang J. EV71 vaccine, an invaluable gift for children. *Clin Transl Immunology* 2014; 3: e28.
10. Xu J, Qian Y, Wang S, et al. EV71: an emerging infectious disease vaccine target in the far east? *Vaccine* 2010; 28: 3516–3521.
11. Tsaoa KC, Huang CG, Huang YL, et al. Epidemiologic features and virus isolation of enteroviruses in Northern Taiwan during 2000–2008. *J Virol Methods* 2010; 165: 330–332.
12. Tan EL, Yong LL, Quak SH, et al. Rapid detection of enterovirus 71 by real-time TaqMan RT-PCR. *J Clin Virol* 2008; 42: 203–206.
13. Wu W, Xu WB, Chen L, et al. Molecular identification and analysis of human enteroviruses isolated from healthy children in Shenzhen, China from 2010 to 2011. *PLoS One* 2013; 8: e64889.
14. Hwang S, Kang B, Hong J, et al. Development of duplex real-time RT-PCR based on Taqman technology for detecting simultaneously the genome of pan-enterovirus and enterovirus 71. *J Med Virol* 2013; 85: 1274–1279.
15. He SJ, Han JF, Ding XX, et al. Characterization of enterovirus 71 and coxsackievirus A16 isolated in hand, foot, and mouth disease patients in Guangdong, 2010. *Int J Infect Dis* 2013; 17: e1025–e1030.

16. Wang C, You A, Tian X, et al. Analysis and solution of false-positives when testing CVA16 sera using an antibody assay against the EV71 virus. *Virus Res* 2013; 176: 33–36.
17. Xu F, Yan Q, Wang H, et al. Performance of detecting IgM antibodies against enterovirus 71 for early diagnosis. *PLoS One* 2010; 5: e11388.
18. Huang YP, Lin TL, Lin TH, et al. Antigenic and genetic diversity of human enterovirus 71 from 2009 to 2012, Taiwan. *PLoS One* 2013; 8: e80942.
19. Zhang J, Dong M, Jiang B, et al. Antigenic characteristics of the complete and truncated capsid protein VP1 of enterovirus 71. *Virus Res* 2012; 167: 337–342.
20. Chen YC, Li C, He D, et al. Antigenic analysis of divergent genotypes human Enterovirus 71 viruses by a panel of neutralizing monoclonal antibodies: Current genotyping of EV71 does not reflect their antigenicity. *Vaccine* 2013; 31: 425–430.
21. Kalish RA, McHugh G, Granquist J, et al. Persistence of immunoglobulin M or immunoglobulin G antibody responses to Borrelia burgdorferi 10–20 years after active Lyme disease. *Clin Infect Dis* 2001; 33: 780–785.
22. Paldanius M, Bloigu A, Alho M, et al. Prevalence and persistence of Chlamydia pneumoniae antibodies in healthy laboratory personnel in Finland. *Clin Diagn Lab Immunol* 2005; 12: 654–659.
23. Kijanka G, Ipcho S, Baars S, et al. Rapid characterization of binding specificity and cross-reactivity of antibodies using recombinant human protein arrays. *J Immunol Methods* 2009; 340: 132–137.
24. Gnjatic S, Wheeler C, Ebner M, et al. Seromic analysis of antibody responses in non-small cell lung cancer patients and healthy donors using conformational protein arrays. *J Immunol Methods* 2009; 341: 50–58.
25. Jiang W, Huang R, Duan C, et al. Identification of five serum protein markers for detection of ovarian cancer by antibody arrays. *PLoS One* 2013; 8: e76795.
26. Lessa-Aquino C, Borges Rodrigues C, Pablo J, et al. Identification of seroreactive proteins of Leptospira interrogans serovar copenhageni using a high-density protein microarray approach. *PLoS Negl Trop Dis* 2013; 7: e2499.
27. Abel L, Kutschki S, Turewicz M, et al. Autoimmune profiling with protein microarrays in clinical applications. *Biochim Biophys Acta* 2014; 1844: 977–987.
28. Xu F, He D, He S, et al. Development of an IgM-capture ELISA for Coxsackievirus A16 infection. *J Virol Methods* 2011; 171: 107–110.
29. Wang SY, Lin TL, Chen HY, et al. Early and rapid detection of enterovirus 71 infection by an IgM-capture ELISA. *J Virol Methods* 2004; 119: 37–43.
30. Yan XF, Gao S, Xia JF, et al. Epidemic characteristics of hand, foot, and mouth disease in Shanghai from 2009 to 2010: enterovirus 71 subgenotype C4 as the primary causative agent and a high incidence of mixed infections with coxsackievirus A16. *Scand J Infect Dis* 2012; 44: 297–305.
31. Lin Y, Wen K, Pan Y, et al. Cross-reactivity of anti-EV71 IgM and neutralizing antibody in series sera of patients infected with Enterovirus 71 and Coxsackievirus A 16. *J Immunoassay Immunochem* 2011; 32: 233–243.
32. Wu TC, Wang YF, Lee YP, et al. Immunity to avirulent enterovirus 71 and coxsackie A16 virus protects against enterovirus 71 infection in mice. *J Virol* 2007; 81: 10310–10315.
33. Zhao Y, Zhang Y, Lin D, et al. Protein microarray with horseradish peroxidase chemiluminescence for quantification of serum a-fetoprotein. *J Int Med Res* 2015; 43: 639–647.