

Research Paper

Expression of enterovirus 71 capsid protein VP1 in *Escherichia coli* and its clinical application

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Abstract

The VP1 gene of enterovirus 71 (EV71) was synthesized, construct a recombinant plasmid pET15b/VP1 and expressed in *E. coli* BL21. The recombinant VP1 protein could specifically react with EV71-infected patient sera without the cross-reaction with serum antibodies of coxsackievirus A16 (CA16), A4, A5, B3 and B5 as well as echovirus 6. In acute and convalescent phases, IgM and IgG antibodies of 182 serum samples were detected by ELISA with recombinant VP1 protein as a coated antigen. The results showed that the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of IgM antibodies in serum samples for the diagnosis of EV71 infection were 90.1, 98.4, 98.8 and 88.7%, respectively; similarly, those of IgG antibodies in serum samples were 82.4, 89.1, 91.5 and 78.1%, respectively. Five of 80 samples (6.25%) from CA16-infected patients were detected positive by ELISA with recombinant VP1 protein in which indicated the cross reactions and 0 of 5 samples from patients infected with other enteroviruses including CA4, CA5, CB3, CB5 and echovirus 6. Therefore, the recombinant VP1 protein of EV71 may provide a theoretical reference for establishing an effective antibody screening of IgM for EV71-infected patients with clinically suspected hand, foot, and mouth disease (HFMD).

Key words: Enterovirus 71, Gene cloning, Recombinant VP1 protein, ELISA.

Introduction

EV71 is one of the most important pathogens in the family of *Picornaviridae* that can cause severe complications, from mild HFMD to severe neurological syndromes, such as encephalitis, pulmonary edema, and even death. Outbreaks of EV71 infection have been reported around the world since 1969.(Melnick *et al.*, 1974; Schmidt *et al.*, 1974; Herrero *et al.*, 2003; Wu *et al.*, 2010; Zhang *et al.*, 2010) It often causes a mild illness, and most patients usually recover quickly. However, in the past decade, a significant increase in EV71 epidemics is observed, and it has emerged as a serious threat to public health throughout the Asia-Pacific region, such as Taiwan, Malaysia and Singapore as well as Guangdong, Hunan, Jiangsu and Fuyang in

China.(Yan *et al.*, 2000; Chan *et al.*, 2003; Herrero,2003; Mao *et al.*, 2010; Zhang,2010; Zhu *et al.*, 2010) EV71 and CA16 infections mainly occur in children under 5 years old. However, patients infected with EV71 are more liable to develop severe complications, including encephalitis, aseptic meningitis, pulmonary edema or haemorrhage, and acute flaccid paralysis. (Iwai *et al.*, 2009; Ooi *et al.*, 2010; Solomon *et al.*, 2010)

EV71 is a small genus of human enterovirus RNA virus family A, and closely related to CA16. EV71 possesses a single-stranded RNA genome of approximately 7400 bp, consisting of a single open reading frame (ORF) flanked by 5'-untranslated regions (5'-UTR) and 3'-untranslated regions (3'-UTR).(Chua *et al.*, 2008) The ORF is expressed as a large polyprotein that can be cleaved into P1, P2, and

P3 regions. The P1 region encodes four structural proteins including VP1, VP2, VP3, and VP4. (Lal *et al.*, 2006) The P2 and P3 regions encode nonstructural proteins, such as proteases 2A, 2B and 3CD, responsible for virus replication and virulence. Variation of capsid proteins, except VP4, is responsible for the antigenic diversity among enteroviruses, but neutralizing epitopes reside mainly on VP1. (Foo *et al.*, 2008)

Traditional detection of EV71 infection is primarily dependent on virus cultivation, serodiagnosis and real-time PCR assays. (Li *et al.*, 2002; Singh *et al.*, 2002; Solomon, 2010) However, virus culture and real-time PCR assays are time-consuming and need special facilities (Rigouan *et al.*, 1998; Zhang *et al.*, 2009; Chen *et al.*, 2011). Early diagnosis can be helpful for the administration of appropriate treatments which may limit the spread of this virus and reduce the mortality of patients. In this study, the entire VP1 gene of EV71 was synthesized and expressed in *E. coli* BL21 (DE3). The capsid VP1, as a natural protein with molecular mass of 36 kDa evaluated by SDS-PAGE, had the desired immunogenicity against EV71 antibody. The aim of this study is to obtain a recombinant VP1 antigen for establishing a rapid serological test for the diagnosis and epidemiological investigation of EV71 infection.

Materials and Methods

Specimen collection

From March to September of 2009, a total of 176 rectal and 176 throat swabs were collected from 176 patients with HFMD under the age of 5 years old enrolled in Changzhou Hospital in China. During acute (0-5 days) and convalescent (14-30 days) phases, 182 serum samples were harvested in duplicates for the detection of IgM and IgG antibodies. 64 control serum samples were collected from healthy children with the mean age of 2.5 ± 1.3 years old. These children showed no disease symptoms and didn't present with a previous history of EV71 and CA16 infection at the time of sample harvesting. In addition, 80 serum samples from CA16-infected patients (0-5 days) with the mean age of 2.5 ± 1.3 years old. The harvested serum samples were stored at -80°C for future use. CA16 serum (horse) was provided by American Type Culture Collection (ATCC). Five serum samples collected from patients infected by coxsackievirus A4 (CA4), coxsackievirus A5 (CA5), coxsackievirus B3 (CB3), coxsackievirus B5 (CB5) and echovirus 6 were gifts from Changzhou Center for Disease Control and Prevention, and Nanjing Medical University. This study was approved by the local ethics committee and all parents/guardians of children were provided with a description of the study and were asked to give informed consents.

Detection of rectal and throat swabs by PCR fluorescence probing assay

Fecal specimens were mixed thoroughly with 5 to 10 volumes of phosphate-buffered saline (PBS) (pH 7.4) to generate homogeneous suspensions. The mixtures were clarified by centrifugation at 13,000 g for 5 min. Viral RNA was extracted from supernatants of fecal suspensions and throat swabs using QIAamp viral RNA Mini kit (QIAGEN, Germany) according to the manufacturer's instructions. The RNA was eluted from the QIAspin column in a final volume of 100 μL of elution buffer and kept at -80°C until further analysis. The PCR fluorescence probing assay reagent (DaAn Gene, China) for EV71 and CA16 were commercially available. The cDNA was generated in a 20 μL of reaction volume for 30 min at 40°C using random primers and SuperScript II reverse transcriptase (Invitrogen, USA) according to the instructions. The EV71 cycling conditions were composed of 5 min at 94°C , followed by 40 cycles with 93°C for 15 s, 55°C for 45 s and 72°C for 1 min, and a final extension cycle at 72°C for 10 min. The primers of EV71/VP1 were designed according to the complete gene sequences (2800-2930 bp) published in GenBank (accession No. AY465356). Forward primer EV71-F: 5'-AAA GGT GGA GCT GTT CAC CTA CAT GCG CTT TGA C-3', reverse primer EV71-R: 5'-AAT CTG GCT TGG GGG CCC CAG GTG GTA CAA-3', and oligonucleotide probe EV71-P: 5'-CCC ACC GGG GAA GTT GTC CCA CAA TTG CTC C-3'. The CA16 cycling conditions were composed of 3 min at 94°C , followed by 40 cycles with 93°C for 15 s, 55°C for 45 s and 72°C for 1 min, and a final extension cycle at 72°C for 10 min. The primers of CA16 were designed according to the complete gene sequences (1909-1970 bp) published in GenBank (accession No. EU262658). Forward primer CA16-F1: 5'-CAT GCA GCG CTT GTG CTT-3', CA16-F2: 5'-CAT GCA ACG ACT GTG CTT TC-3'. Reverse primer CA16-R1: 5'-CAC ACA ATT CCC CCG TCT TAC-3', and CA16-R2: 5'-CAT AAT TCG CCC GTT TTG CT-3'.

Virus isolation and identification

Clinical specimens including rectal swabs and throat swabs were inoculated into rhabdomyosarcoma (RD) cells and human laryngeal carcinoma (Hep-2) cells (Chinese Academy of Sciences Cell Bank of Type Culture Collection, CBTCCAS) for the isolation of EV71. Cytopathic effects (CPE) were examined under an inverted microscope after 2 to 7 days. Enterovirus strains were identified by immunofluorescence test using EV71 monoclonal antibody (Chemicon International Inc).

Construction of expression vector

The nucleotide sequence for capsid protein VP1 gene of EV71 has been deposited in the GenBank database and synthesized according to the accession number of

AY465356 (Human enterovirus 71 strain SHZH03). The amino acid sequence of recombinant VP1 fusion protein was shown in Figure 1. The *NdeI* and *XhoI* restriction endonuclease (Promega, USA) sites in primers were then introduced to allow ligation of the entire VP1 cDNA into pET15b (Novagen, Germany), a prokaryotic expression vector containing an amino-terminal histidine tag.

The expression and purification of VP1 recombinant proteins

The synthesized VP1 gene was verified by DNA sequencing and restriction endonuclease digestion before expression. Competent *E. coli* BL21 (DE3) cells (Novagen, Germany) were transformed with pET15b expression vector harboring VP1 cDNA (pET-VP1). *E. coli* cells were grown in shaker flasks with LB broth medium containing 50 µg/mL ampicillin at 37 °C until optical density (OD) of 0.6 was reached. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the LB medium at the final concentration of 1 mM to induce the expression of His-VP1 fusion protein. At 0, 1, 2 and 4 h after induction, the cells were harvested and centrifuged at 3,200 g for 10 min and washed once with PBS. Subsequently, the cells were subjected to rapid freeze-thaw treatment at -80 °C twice and then resuspended in PBS buffer. In addition, cell membranes were disrupted by sonication at 180 Hz, and then the precipitates and supernatants were collected at 10,310 g for 30 min at 4 °C. The Ni²⁺ chromatography columns (GE Healthcare, USA) were washed with 25% ethanol and double-distilled water, and then equilibrated with 30 mL buffer. The supernatants were filtered by a 0.22 µm-pore-size filter and flow-through chromatography column. 20 mL of buffer containing 500 mmol / L imidazole for elution and collecting 1 mL / tube each. 5 µL of samples were taken from each tube and analyzed by 15% SDS-PAGE. The purified protein desalination was completed by HiTrap Desalting column (GE Healthcare, USA) according to the manufacturer's instructions.

Western blot analysis

The purified VP1 proteins were dissolved in SDS-PAGE sample buffer and then heated at 95 °C for 5 min. The denatured proteins were subjected to SDS-PAGE (15% polyacrylamide) separation and then transferred to the nitrocellulose membrane (Generay, Shanghai) by electroblotting for 1 h at 1 Amp. After blocking with diluted Tris-buffered saline containing 0.05% Tween-20 (TBST) and 5% skim milk for 1 h, rabbit anti-His-tag polyclonal antibody (Santa Cruz Biotechnology, CA) was added at a dilution ratio of 1:1,000 in TBST solution at 37 °C for 1 h. After washing with TBST, goat anti-rabbit IgG (H+L) conjugated with horseradish peroxidase (Santa Cruz Biotechnology, CA) as secondary antibody, was added at the dilution ratio of 1:1,000 and incubated at 37 °C for 1 h, and then incubated with 4-chloro-1-naphthol solution for 5-10 min. The cell culture of pET15b/VP1-BL21 without induction was used as the negative control.

The serum cross-reaction of CA16 and other non-EV71 enteroviruses

The purified VP1 protein was examined by Western blot. IgM and IgG positive serum sample of CA16 and 5 serum samples collected from patients infected by other enteroviruses (CA4, CA5, CB3, CB5, and echovirus 6) were diluted at a ratio of 1:500. Goat anti-human conjugated with HRP (Santa Cruz Biotechnology, CA) was used as the secondary antibody. IgM and IgG positive serum samples of EV71 were used as the positive controls.

Detection of EV71 IgG or IgM antibody by ELISA with recombinant VP1 protein

A 96-well microtiter plate was coated with 100 µL of recombinant VP1 protein (10 µg/mL) in carbonate coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). The plates were incubated at 4 °C overnight, and then washed twice with phosphate-buffered saline containing Tween-20 (PBS-T). The plates were incubated with 1% bovine serum albumin in PBS for 2 h at room temperature to prevent

	<u>His Tag</u>	<u>VP1</u>	
1	MGSSHHHHHSSGLVPRGSGDRVADVI	ESSIGDSVSRAL	TQALPAPTQNTQVSSHRLDT 60
61	GKVPALQAAEIGASSNASDESMIETRCV	LNSHSTAETTLDSFFSRAGLVGEIALPLEGTP	120
121	NPNGYANWDIDITGYAQMRRKVELFTDMR	FDAEFTFVACIPTGEVVPQLLQYMFVPPGAP	180
181	KPDSRESLAWQTATNFSVFKLSDP	PAQVSVPFMSPASAYQWFYDGYPTFGEHKQEKDFE	240
241	YGACPNMMGIFSVRTVGTSKSKYFLV	VRIYMRMKHVRWILCPMRNQNYLFKANPNYAG	300
301	NSIKPTGASRAAITT	315	

Figure 1 - The amino acid sequence of recombinant VP1 protein.

non-specific binding. Totally 100 μ L of 1:300 diluted serum sample was added to each well and incubated at 37 °C for 1 h. The plate was washed for 4 times with PBS-T, followed by horseradish peroxidase conjugated goat anti-human IgG or IgM (1: 2,000 dilution, Santa Cruz Biotechnology, CA). The reaction was developed by 100 μ L of 3, 3', 5, 5'-tetramethyl benzidine (TMB) substrate, and then terminated by 100 μ L of 2 M H₂SO₄. The OD at 450 nm were determined.

Statistical analysis

The OD values of IgM and IgG antibodies between healthy objects and HFMD patients were compared by unpaired Student *t*-tests. A significant difference was considered at *P* value less than 0.05. The sensitivity, specificity, PPV and NPV were evaluated for the diagnostic value of anti-EV71 IgM and IgG antibodies. The diagnostic efficiency of EV71 infection between IgM and IgG antibodies was assessed by receiver operating characteristic curve (ROC) analysis.

Results

PCR fluorescence probing assay

Rectal and throat swabs were collected from 176 patients with HFMD. Of these, 91 patients were detected positive by PCR fluorescence probing assay, as well as further verified by virus culture, immunofluorescence test and clinical manifestations.

Construction of recombinant EV71 pET15b/VP1 plasmid for protein expression

In order to construct expression plasmid of pET15b/VP1 according to VP1 gene sequence, the VP1 gene was amplified and sequenced with the expected length of 891-bp VP1 sequence, and then cloned in pET15b vector digested by *NdeI* and *XhoI* to obtain recombinant pET15b/VP1 plasmid for protein expression (Figure 2).

Expression of recombinant VP1 protein

The recombinant pET15b/VP1 plasmid was transformed into *E. coli* BL-21 (DE3) strain grown on LB medium containing ampicillin at 37 °C and the expression of VP1 protein was induced by 1 mM IPTG. The VP1 was produced as a fusion protein with a six-histidine tag at its amino terminus. A band with an approximately 36 kDa from the cell culture induced by IPTG in 15% SDS-PAGE was observed, which is consistent with the expected molecular mass of VP1 fusion protein (Figure 3).

Detection of purified VP1 protein

The purified protein was confirmed by Western blot. The result showed that the recombinant VP1 protein revealed a specific band with expected molecular mass of 36 kDa, which was specifically recognized by anti-His poly-

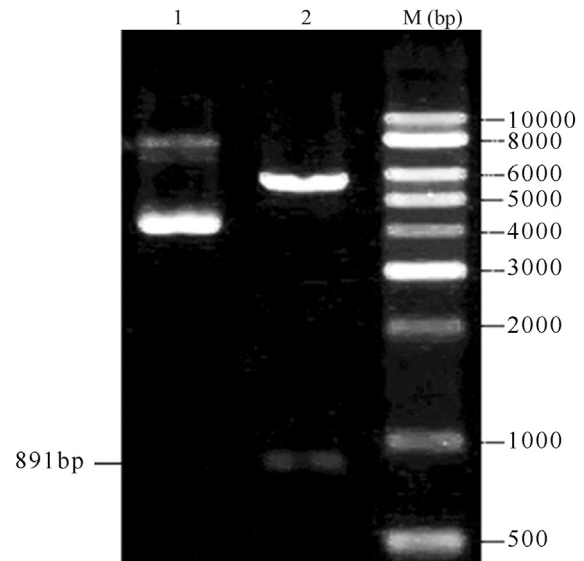


Figure 2 - The electrophoresis diagram of pET-15b/VP1 and Digestion with *NdeI* and *XhoI*. M: Marker, DNA ladder. Lane 1: pET-15b/VP1; Lane 2: pET-15b/VP1 digested by *NdeI* and *XhoI*.

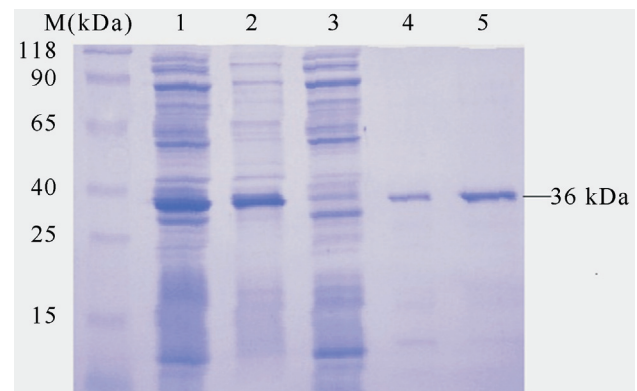


Figure 3 - 15% SDS-PAGE analysis of VP1 expression. M: Protein marker (kDa); Lane 1: Recombinant VP1 protein in supernatant; Lane 2: Recombinant protein VP1 in precipitate; Lane 3: VP1 without IPTG induction; Lane 4: Purified VP1 protein; Lane 5: VP1 protein after desalting.

clonal antibody. However, in the negative control, empty vector pET-15b could not be induced to express VP1 protein so that it did not have a specific band shown in Western blot (Figure 4).

The serum cross-reaction of EV71 and other enteroviruses

In order to evaluate the cross-interaction, Western blot analyses for recombinant VP1 antigen using serum samples from patients with EV71 and CA16 infections, as well as other 5 enteroviruses including CA4, CA5, CB3, CB5 and echovirus 6 were conducted. The experiments were repeated three times and the results showed that there was no cross-reaction between recombinant VP1 protein and other positive enterovirus serum samples (Figure 5).

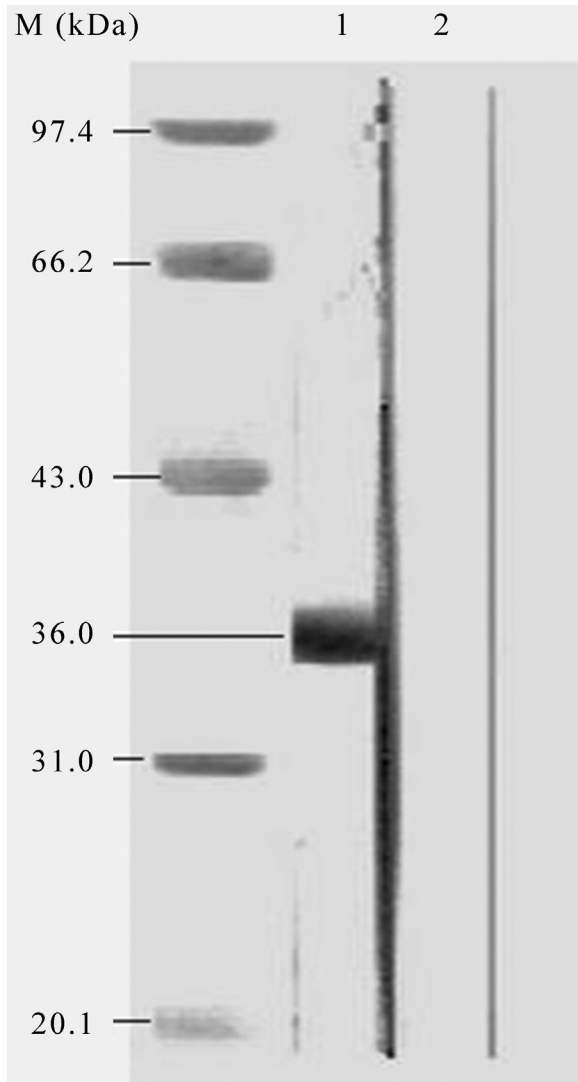


Figure 4 - Western blot analysis of purified recombinant VP1 protein. M: Protein marker (kDa); Lane 1: purified VP1 protein; Lane 2: pET-15b vector control.

Detection of patient sera with HFMD

The serum samples from 91 patients and 64 healthy subjects were determined by ELISA with purified VP1. However, all serum samples tested in this study were negative for rheumatoid factor and antinuclear factor. There were no cross-reaction between recombinant VP1 protein and CA16 positive serum samples as well as 5 samples from patients infected with other enteroviruses. The OD values of anti-EV71 IgM and IgG in patients were significantly elevated when compared with that of healthy subjects ($p < 0.001$). Compared with PCR fluorescence probing assay, virus culture, immunofluorescence test and clinical manifestation, ROC was determined by OD values of EV71 IgM and IgG antibodies in patients and controls, and their areas under curve (AUC) were 0.958 and 0.929, respectively (Figure 6). If a cutoff value of 0.0955 was

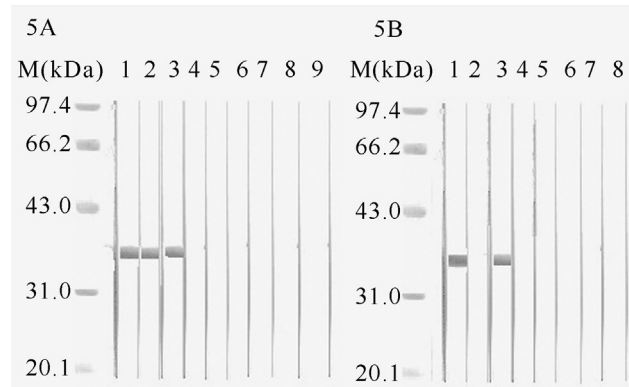


Figure 5 - Western blot analysis of EV71 and other enteroviruses. M: Protein marker (kDa). (5A) Western blot analysis using goat anti-human IgG antibody as the secondary antibody. Lanes 1-3 represent the immunoblotting of serum samples from patients with previous infection of enterovirus 71; Lanes 4-9 were the immunoblotting of serum samples from patients with infections of CA16, CA4, CA5, CB3, CB5 and echovirus 6. (5B) Western blot analysis using goat anti-human IgM antibody as the secondary antibody. Lanes 1-3 represent the immunoblotting with serum samples from patients with asymptomatic enterovirus 71 acute infection, and lanes 4-8 were the immunoblotting of serum samples from patients with infections of CA16, CA4, CA5, CB3 and echovirus 6.

adopted, EV71 IgM antibody had a high specificity (98.4%) and a sensitivity (90.1%), as well as PPV (98.8%) and NPV (88.7%). Among 91 serum samples from patients with EV71 infection in acute phase, 9 serum samples were detected as IgM negative but positive for virus culture. Similarly, among 64 healthy subjects, 1 serum sample was tested positive for IgM antibody (OD = 0.147), however, it was confirmed to be a false positive by PCR fluorescence probing assay, virus culture and indirect immunofluorescence tests. Among 80 samples from CA16-infected patients, 5 were detected IgM antibody positive, which showed that the cross reactions existed. In addition, 5 samples from patients infected with other enteroviruses were negative. Therefore, the sensitivity, specificity, PPV and NPV of the VP1 against IgG were 82.4, 89.1, 91.5 and 78.1% at a cutoff value of 0.135 (Table 1).

Discussion

HFMD, a common illness in children, can be caused by many human enteroviruses, such as coxsackievirus A4, A5, A8, A10, A16, B3 and EV71 (Yoke-Fun and Abu-Bakar, 2006; Wu, 2010) Human EV71 and CA16 are two major causative agents of HFMD. EV71 and CA16 infections manifesting as HFMD and herpangina are clinically indistinguishable, but EV71 infection is more frequently associated with serious neurological complications and fatalities. (Nagata *et al.*, 2004; Li *et al.*, 2005; Xu *et al.*, 2010) Thus, rapid discrimination between EV71 and CA16 is highly desired during HFMD outbreaks. EV71 genome encodes VP1, VP2, VP3 and VP4 capsid proteins. VP1, VP2 and VP3 are exposed to the virus surface, while VP4 is embedded in the virus capsid. In particularly, VP1 protein is

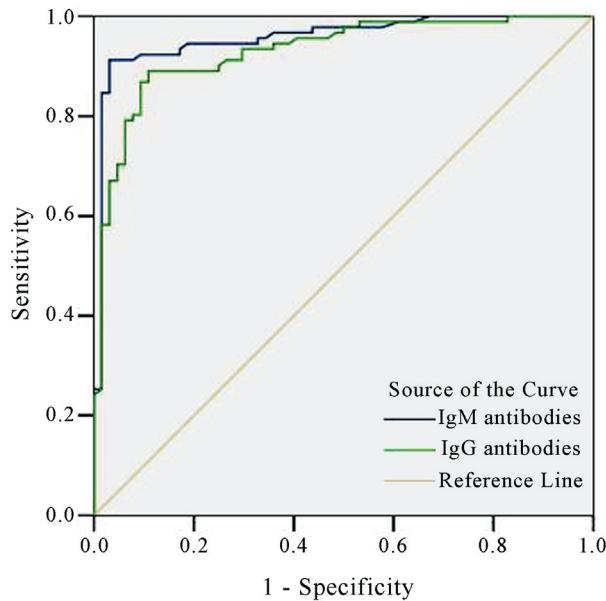


Figure 6 - ROC curves of IgM and IgG antibodies for the detection of EV71 infection. IgM antibody had a high specificity (98.4%) and a sensitivity (91.2%), as well as PPV (98.8%) and NPV (88.7%) at a cutoff value of 0.0955. However, the sensitivity, specificity, PPV and NPV of the VP1 against IgG were 82.4, 89.1, 91.5 and 78.1% at a cutoff value of 0.135.

the major neutral epitope of EV71. VP1 gene is by far becoming the major target for molecular epidemiology research and vaccine. (Chen *et al.*, 2008; Zhang & Lu, 2010) In this study, whole VP1 gene of EV71 was cloned and expressed in *E. coli*. The VP1 fusion protein with a histidine tag had an estimated molecular mass of 36 kDa, and was expressed in *E. coli* with IPTG induction. The immunogenicity of purified VP1 protein was tested by Western blot analysis using human serum samples containing IgG or IgM antibodies of CA16, CA4, CA5, CB3, CB5 and echovirus 6. Experimental results indicated that, although the serum samples from the patients infected with enteroviruses had a high titer of IgM and IgG, these serum samples did not react with recombinant VP1 fusion protein.

Traditional laboratory diagnosis for EV71 is by cell culture followed by neutralization tests with serotype-specific antisera. (Castro *et al.*, 2005) However, it is time-

consuming and usually needs several weeks. Recently, reverse transcription PCR (RT-PCR) and real-time PCR assays have been used for EV71 detection. (Chen *et al.*, 2006; Tan *et al.*, 2008; Xiao *et al.*, 2009) Unfortunately, these methods require expensive and special equipments and trained personnel, and can not be applied in primary healthcare agencies. Therefore, the laboratory diagnosis of EV71 infection is not satisfactory. In addition, because EV71 and CA16 infections in patients have similar clinical symptoms and both nucleotide sequences of the genomes have high homology (VP1 homology up to 67%). (Ho, 2000; Li, 2005; Iwai, 2009) it is very important to distinguish EV71 and coxsackievirus infections to reduce complications and mortality in early diagnosis of EV71 infection. (Xu *et al.*, 2010) In the present study, we have developed an efficient, rapid and inexpensive diagnostic kit for EV71 infection through purified VP1 fusion protein to detect IgM or IgG antibody. Among 91 serum samples from patients with EV71 infection in acute phase, 82 serum samples were tested as IgM positive, while 9 serum samples were negative. These 8 infected patients were in the window period so that the IgM serum titer was very low, thus leading to false negative results. On the other hand, among 64 healthy controls, only 1 serum was tested as IgM positive, but this sample was confirmed as false positive result by virus culture and indirect immunofluorescence tests. Nevertheless, this control sample cannot be ruled out as a positive case for EV71. In convalescent phase, 80 serum samples were tested as IgG positive and 11 serum samples were negative among totally 91 tested serum samples. In contrast, among 64 healthy controls, 7 serum samples were tested as IgG positive, which may be due to previous subclinical infection. IgM antibody had a high specificity (98.4%) and sensitivity (90.1%), while the sensitivity and specificity of IgG were 89.1 and 82.4%, respectively. It is confirmed that IgM antibody detection can be used for diagnosis of acute or primary infection while IgG antibody can only be used for determination of host immune status. Five of 80 samples (6.25%) from CA16-infected patients were detected positive by ELISA with recombinant VP1 protein in which indicated the cross reactions and 0 of 5 samples from patients infected with other enteroviruses including CA4, CA5, CB3, CB5 and echovirus 6.

Table 1 - ELISA analysis of recombinant VP1 protein using serum samples from 91 EV71-infected patients, 80 CA16-infected patients and 64 healthy persons.

Results of ELISA	IgM antibodies				IgG antibodies		
	EV71 No.	CA16 No.	Control No.	Total	Tested No.	Control No.	Total
Positive	82	5	1	83	75	7	82
Negative	9	75	63	72	16	57	73
Total	91	80	64	155	91	64	155

Tested: EV71-infected patients. Control: healthy persons. All patients infected with EV71 or CA16 were confirmed by PCR fluorescence probing assay, virus culture, immunofluorescence test, and combined with clinical manifestations.

When rheumatoid factor or antinuclear factor is present in serum, the detection of specific IgM antibody commonly leads to false positive results by using ELISA. (Shih *et al.*, 2000) Therefore, IgM detection has a problem in early infectious diseases because transient production of rheumatoid factor is often observed at the beginning of many infections. We should saturate the sample with aggregated IgG or Staphylococcal protein A to pre-treat sera for preventing rheumatoid factor or antinuclear factor binding. (Renaudineau *et al.*, 2005) In this study, these factors weren't observed in serum samples from patients and healthy subjects.

Taken together, the results of this study suggest that the recombinant VP1 protein is a suitable antigen for the detection of IgM antibody in the early serodiagnosis of EV71 infection.

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