Development of a high-throughput assay for measuring serum neutralizing antibody against enterovirus 71

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ABSTRACT

Enterovirus 71 (EV71) is the main etiologic agent of hand, foot, and mouth disease (HFMD) and causes frequently severe neurological complications and mortality in young children. The serum neutralizing antibody response is the major indicator of EV71 infection and protective immunity. The current serum neutralization test based on inhibition of cytopathic effect (Nt-CPE) requires manual microscopic examination, which is time-consuming and labor-intensive. In this study, a high-throughput neutralization assay which employs enzyme immunoassay for detecting growth of EV71 in Rhabdomyosarcoma (RD) cells and measuring serum neutralizing antibody (Nt-EIA) against EV71 was developed. RD cells infected with 100 $TCID_{50}$ of EV71 for 36–42 h had the best performance and were selected for Nt-EIA. One hundred and twenty human sera (59 negative sera, 61 positive sera) were measured for EV71 neutralization antibody titers by Nt-CPE and Nt-EIA. Neutralization antibody titers against EV71 determined by Nt-EIA had a high sensitivity (100%), specificity (94.9%) and agreement (97.5%) by a qualitative comparison with Nt-CPE. In the quantitative comparison, the correlation coefficient between Nt-EIA and Nt-CPE was 0.91 after log transformation. Overall, the Nt-EIA is a suitable alternative assay for the quantitation of EV71 neutralizing antibody to EV71.

1. Introduction

Human enterovirus 71 (EV71) belongs to Picornaviridae and is a member of human enterovirus species A. EV71 is classified genetically into 3 genogroups (A, B, and C) and 11 genotypes (A, B1–B5, C1–C5). The clinical spectrum of EV71 infection ranges from asymptomatic infection, mild hand–foot–month diseases (HFMD), herpangina, to severe cases with neurological and cardiopulmonary complications. EV71 was first isolated in California, USA, in 1969. Since then, EV71 has been isolated globally and causes life-threatening outbreaks in young children in Asia (Bible et al., 2007). In Taiwan, a nation-wide EV71 epidemic occurred in 1998. Subsequently, EV71 has become an endemic disease and has caused several nation-wide outbreaks (Huang et al., 2008; Lin et al., 2003).

Serum neutralizing antibody is the major indicator of EV71 infection and protective immunity. The neutralization test based on inhibition of cytopathic effect (CPE) is a standard method recommended by the world Health Organization (WHO) for measuring neutralizing antibody against polioviruses (WHO, 1997) and was also applied widely to determine the neutralizing antibody titers against EV71 (Chang et al., 2002; Kung et al., 2007). The traditional CPE-based neutralization test (Nt-CPE) does not require special reagents but is labor-intensive, subjective and time-consuming (at least 4 days). Therefore, this method is not suitable for mass screening of protective immunity in seroepidemiological studies. Recently, simplified neutralization tests that employ enzyme immunoassay (EIA) to detect virus, have been applied in detection of neutralizing antibody against multiple viruses including respiratory syncytial virus, mumps virus, Jatoba virus, hantavirus, influenza virus, rotavirus and measles virus (Anderson et al., 1985; Benne et al., 1994; Figueiredo and Da Rosa, 1988; Horling et al., 1992; Lee et al., 1999; van Tiel et al., 1988; Ward et al., 1996). In this study, a high-throughput assay was developed for measuring serum neutralizing antibodies against enterovirus 71 based on enzyme immunoassay.

2. Materials and methods

2.1. Viruses and cells

EV71 strain TW/E59/2002 (B4 genotype) was grown in Rhabdomyosarcoma (RD) cells and used for all tests in this study. To estimate viral infectivity titers, EV71 was diluted serially 10-fold and incubated with RD cells on 96-well microtiter plates. CPE was observed using an inverted microscope after an incubation period

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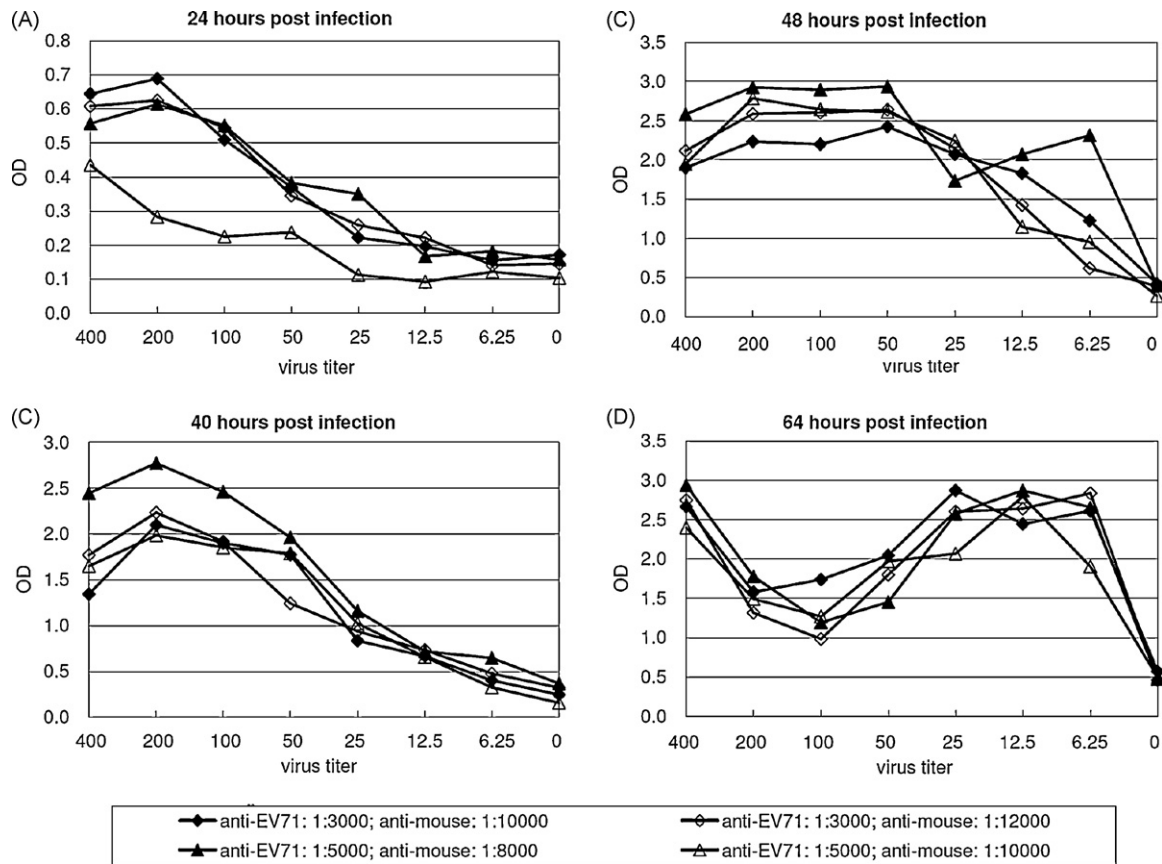
Fig. 1. The OD values of EV71 at different incubation times and antibody dilutions. EV71 was diluted serially 2-fold from 400 TCID$_{50}$/well and cultured in RD cells. The OD value at each point was the mean OD for 3 wells. (A) Incubation for 24 h. (B) Incubation for 40 h. (C) Incubation for 48 h. (D) Incubation for 64 h.

of 4 days. The 50% tissue culture infectious doses (TCID$_{50}$) of EV71 were calculated by the method of Reed and Muench (1938).

2.2. Nt-CPE

Sera were heat-inactivated at 56 °C for 30 min and diluted serially 2-fold from 1:8 to 1:512 and mixed with an equal volume (50 μl) of virus working solution containing 100 TCID$_{50}$/well of EV71 at 37 °C for an hour in 96-well microtiter plates. RD cells at 3×10^4/well were added and incubated for 4 days. Sera samples were tested in triplicate and the neutralization titers were read as the highest dilution that completely inhibited CPE in over 50% of wells. A positive control serum with a known titer was included in each run.

2.3. Nt-EIA: determination of incubation time

To determine the optimal incubation time for the Nt-EIA, EV71 was diluted serially 2-fold from 400 TCID50/well and incubated with RD cells for 24, 40, 48, and 64 h, separately. After incubation, the plates were fixed with cold (4 °C) acetone 80% (vol:vol) and air dried. For the EIA test, air-dried plates were rehydrated by phosphate-buffered saline containing 0.05% Tween-20 (PBS:T). To detect EV71, mouse monoclonal antibody against EV71 (MAB 979, Millipore Corporation, Temecula, CA, USA) was used as the primary antibody and horseradish peroxidase (HRP) labeled anti-mouse IgG (Jackson Immuno Research, Pennsylvania, USA) was used as the secondary antibody diluted in PBS:T containing 3% bovine albumin. The optical densities (ODs) were read at 450 and 650 nm using TMB (3,3%, 5,5%-tetramethylbenzidine, ImmunoBioScience, Washington, USA) for color development.

2.4. Nt-EIA

Sera were heat-inactivated at 56 °C for 30 min, diluted serially 2-fold and mixed with an equal volume (50 μl) of virus working solution and incubated with a suspension of RD cells. After incubation, the presence of EV71 was detected by indirect EIA as described above. On each plate, 6 wells of virus control and 6 wells of cell controls were included. A virus-positive well was defined as OD values >3-fold of the mean OD values of the cell control wells. All serum dilutions were tested in triplicate and the neutralization titers were read as the highest dilution that completely inhibited virus growth in over 50% of wells.

2.5. Comparison between Nt-EIA and Nt-CPE

For comparison, 120 sera with known Nt-CPE titers (59 negative and 61 positive) were selected from pregnant women and neonates used in a previous study (Luo et al., 2009). The starting dilution of sera was 1:8 and seropositivity was defined as antibody titers ≥8. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of Nt-EIA were calculated by using Nt-CPE as the 'gold standard'. In addition, a kappa statistic was used to evaluate the agreement between Nt-EIA and Nt-CPE (Fleiss, 1981). A kappa statistic of 1 indicates perfect agreement, while a statistic of 0 indicates no agreement above that expected by chance. Nt-CPE positive sera were included for quantitative comparison. The correlation coefficient and the ratio between neutralizing antibody titers measured by Nt-EIA and Nt-CPE were calculated. Statistics were performed using the SAS 9.1.3 program (SAS Institute Inc., USA).

Table 1
Qualitative comparison between Nt-EIA and Nt-CPE.

Nt-CPE titer	Nt-EIA titer <8	Nt-EIA titer ≥8	Sensitivity % (95%CI)[c]	Specificity % (95%CI)	PPV %[a] (95%CI)	NPV %[b] (95%CI)	Agreement % (95%CI)	Kappa (95%CI)
<8	56	3	100	94.9	95.3	100	97.5	0.95
≥8	0	61	(94.1–100)	(85.9–98.9)	(86.9–99.0)	(93.6–100)	(91.9–100)	(0.894–1)

[a] PPV: positive predictive value.
[b] NPV: negative predictive value.
[c] 95%CI = 95% confidence interval.

3. Results

3.1. Determination of the incubation time

After incubation for 24 h, there was no apparent CPE but virus was detected by EIA using 100 $TCID_{50}$/well of EV71. However, the OD values at 24 h were low (Fig. 1A) compared to the OD values of the cell control wells (no virus added). The OD values with higher virus inputs (>100 $TCID_{50}$/well) decreased after incubation for more than 48 h (Fig. 1C and D) because of extensive CPE and cells detached from the plates. As shown in Fig. 1B, there is a good dose–response relationship between OD values and virus inputs at 40 h post-infection (Fig. 1B) and this incubation time and virus inputs of 100 $TCID_{50}$/well were used in the subsequent tests.

3.2. Determination of the concentrations of detection antibodies for EIA

Two concentrations of primary and secondary antibodies were used to detect EV71. At the incubation period of 40 h, OD values for the cell control wells were not greater than 0.4 in all of the combinations tested and the combination of anti-EV71 monoclonal antibody at 1:5000 dilution and anti-mouse IgG antibody at 1:8000 dilution provided the highest OD values (Fig. 1B). Therefore, these two dilutions were used in subsequent tests.

3.3. Qualitative and quantitative comparison between Nt-CPE and Nt-EIA

The sensitivity, specificity, PPV and NPV of Nt-EIA were 100%, 94.9%, 95.3% and 94.9%, respectively (Table 1). The agreement was 97.5% and the kappa statistic was 95.0% between Nt-EIA and Nt-CPE (Table 1). Disagreement was observed in only 3 sera that were Nt-CPE negative and Nt-EIA low positive (1:8). Only 1 of 120 sera had >2-fold difference between antibody titers tested by these two methods (Table 2). In the analysis of the quantitative correlation using the 61 sera with Nt-CPE and Nt-EIA titers greater than 1:8, a correlation coefficient of 0.91 after log2 transformation was observed (Fig. 2).

Table 2
Distribution of neutralizing antibody titers measured by the Nt-CPE and Nt-EIA in 120 sera.

	Number	Nt-EIA titer <8	8	16	32	64	128	256	512
Nt-CPE titer	<8	**56**	3						
	8		**7**	4					
	16		1	**9**	4	1			
	32				**5**	8			
	64				3	**1**	3		
	128					2	**6**	3	
	256							**0**	4

4. Discussion

Nt-CPE is the standard method for measurement of protective antibodies, for laboratory diagnosis of EV71 infection, and for the evaluation of vaccine-induced immunity. The cost of Nt-CPE is low, but requires at least 4 days to complete with manual microscopic examination and special expertise and there may be subjective variations among operators and laboratories. In this study, the Nt-EIA measured neutralization titers within 48 h and using the ELISA method, which is both objective and easy to standardize in different laboratories. Importantly, the Nt-EIA provided high sensitivity (100.0%), specificity (95.3%) and agreement (97.5%) in a qualitative comparison with Nt-CPE. In a quantitative comparison, the correlation coefficient between Nt-EIA and Nt-CPE was 0.91 after log transformation.

Phylogenetically, EV71 is classified into 3 genogroups (A, B and C). In this study, only the B genogroup was tested by Nt-EIA. Notably, the monoclonal antibody (MAB979, Millipore) used to quantify virus growth also detects A and C genogroups (our unpublished data; Landry et al., 1995). Therefore, the Nt-EIA may be applied to all genogroups of EV71 viruses.

Neutralization titers with positive control sera varied within 2–4-fold by the Nt-EIA and the Nt-CPE assays, indicating high precision. High variability in neutralization titers measured among different laboratories has been documented for other viruses, such as influenza virus (Stephenson et al., 2009), and Japanese Encephalitis virus (Ferguson et al., 2008). International standard sera with well-defined titers are needed to reduce variation and increase comparability of neutralization titers for EV71 in different laboratories.

Development of vaccines to protect against EV71 disease is a public health priority in certain countries in Asia. Similar to vaccines developed against poliovirus, neutralizing antibody responses will be an important indicator for evaluation of the immunogenicity of different EV71 vaccine candidates. In addition, the Nt-EIA provides a robust method to coordinate global EV71 surveillance in different laboratories.
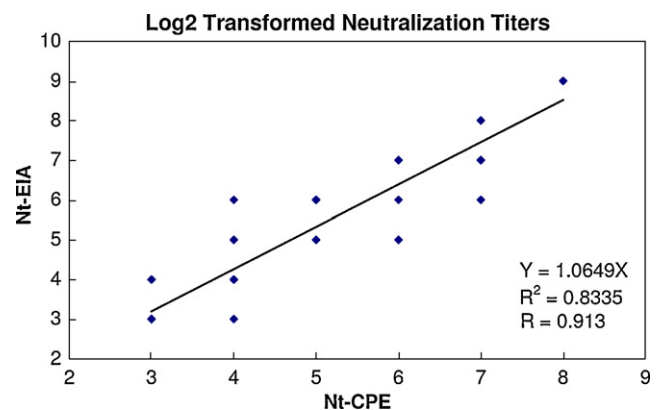

Fig. 2. Correlation between Nt-CPE and Nt-EIA for EV71 neutralizing antibody titers. Neutralization titers were measured by Nt-CPE and Nt-EIA methods and log2 transformed. Only sera with neutralization titers >8 were included.

Acknowledgments

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