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A rapid and quantitative assay for measuring neutralizing antibodies of Coxsackievirus B3

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A B S T R A C T

Coxsackievirus B3 (CVB3) infection has been found to account for an increasing proportion cases of hand, foot and mouth disease (HFMD) in recent epidemiology studies. CVB3 is a single stranded, non-enveloped RNA virus and the infection can cause prominent health threat to pre-school children. Here, by taking approaches of reverse genetics, we established a single-round infection system for CVB3. The pseudovirus was produced by sequential transfection of CVB3 capsid expresser plasmid and CVB3 replicon RNA bearing firefly luciferase as a reporter. The CVB3 pseudovirus system was used for quantifying neutralizing antibody (NtAb) levels of 720 human serum samples and showed superior specificity and sensitivity comparing traditional cytopathic effect (CPE) assay. Furthermore, we compared the seroprevalence of CVB3 NtAbs in pre-school children and healthy adults, and found that only 11.94% of pre-school children were NtAbs positive which suggested that most children were naive to CVB3 infection; while there is much higher positive rate in adults (60%) indicating that most adults have experienced CVB3 infection during childhood. This rapid and quantitative assay greatly facilitates evaluating the level of NtAbs against CVB3 in populations and will help to advance CVB3 vaccine development.

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1. Introduction

Coxsackievirus B3 (CVB3), a member of Coxsackie B viruses which belong to the genus *Enterovirus* within the family *Picornaviridae*, is an important pathogen causing aseptic meningitis, myocarditis and acute pancreatitis in severe cases. CVB3-associated aseptic meningitis have been reported in China (Tao et al., 2012; Tseng et al., 2007; Wong et al., 2011), and CVB3-associated hand foot and mouth disease (HFMD) cases have also been noticed in National Notifiable Diseases Surveillance System (NNDSS) for HFMD. However there is still no effective CVB3 vaccine or antiviral drugs available.

Neutralizing antibodies (NtAbs) elicited after viral infection or vaccination conveyed humoral protection to the population and are essential to viral defense. Thus quantification of NtAbs would be an important criterion in vaccine evaluation as well as the assessment of group protection in human populations before or after vaccination. There are traditional neutralization assay based on inhibition of cytopathic effect (CPE) for viruses that can cause CPE effect on susceptible cell lines *in vitro* including Enterovirus 71 (EV71) (Chang et al., 2002), Coxsackievirus A16 (CA16) as well as CVB3. However, CPE methods usually take 5–7 days of inoculation to let plaques develop and are labor-intensive. Modified enzyme-linked immunosorbent spot assays which significantly reduced the duration time still have its shortcomings, such as biosafety concerns raised by live viruses, limited sensitivity, and semi quantitative results due to poor linearity (Huang et al., 2010; Yang et al., 2014). However, pseudovirus based neutralization assays have distinct advantages, as single-round infection ensures superior biosafety and luciferase reporter enables sensitive quantification (Bentley et al., 2015). Pseudovirus based neutralization assays have been widely used to measure neutralizing antibodies for enveloped viruses, such as Human Immunodeficiency virus (HIV) (Montefiori, 2005), Influenza virus (Tsai et al., 2009), Severe Acute Respiratory Syndrome coronavirus (SARS-coV) (Li et al., 2003; Sui et al., 2004). Most pseudoviruses of enveloped viruses are utilizing optimized lentivirus or retrovirus pseudotyping system, while there is no common pseudotyping system for nonenveloped viruses.

Application of reverse genetics in picornavirus has enable us to get full length infectious viral clones, some pseudoviruses have been made successfully by trans-encapsidation, such as Polio virus

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(Jia et al., 1998), EV71 (Chen et al., 2012), CA16 (Jin et al., 2013). Thereby pseudovirus based neutralizing assays have been successfully developed for EV71 (Wu et al., 2013), CA16 (Jin et al., 2013). However there no efficient pseudotyping system for CVB3 and current assays for detecting neutralizing antibodies against CVB3 are CPE based assays and newly reported ELISA assay (Yang et al., 2014).

In this work, we first established a robust pseudotyping system for CVB3, then developed a neutralizing assay based on this system and verified its suitability in measuring neutralizing antibodies in human serum samples from clinical researches for CVB3. Besides, we used this assay to perform a small scale investigation on CVB3 seroprevalence in Chinese population and evaluated potential threat caused by CVB3 to current HFMD disease control.

2. Materials and methods

2.1. Cell lines, antibodies, virus and antisera

Human embryonic kidney cell (HEK)-293T, Human rhabdomyosarcoma (Porter et al.) cells, Hela cells were maintained in Dulbecco's modified essential media (DMEM) [Life technologies] containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Life technologies) at 37 °C in 5% CO_2. Vero cells were maintained in Minimum Essential Media (MEM) [Life technologies] containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in 5% CO_2. Donkey anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate was purchased from Life technologies. CVB3 strain 112 (DH16G/JS/2012, Genbank accession NO. KP036480) was isolated from a HFMD patient.

Anti-polio virus serum was WHO standard antisera against Polio virus type 1 (Sabin), goat anti-G10/CA16 serum (NT titer 1280) was a gift from the Institute of Medical Biology, Chinese Academy of Medical Science; mouse anti-FY523/EV71 serum was raised by immunization with inactivated EV71 virus FY523, isolated from a patient in Fuyang city during the HFMD epidemic of 2007 in China (Genbank accession no. EU703812, subtype C4); mouse anti-Coxsackievirus B5 (CVB5) serum was raised by immunization with inactivated CVB5 strain, isolated from a three-year-old patient diagnosed with herpangina in Pizhou city in China; And mouse anti-Hepatitis A virus (HAV) was raised by immunization with HAV vaccine produced by Sinovac Biotech Co., Ltd.

2.2. Clinical serum samples

720 serum samples were collected from pre-school children (14 months to two years old) who were enrolled in a Phase III clinical trial on EV71 inactivated vaccine (ClinicalTrials.gov Identifier: NCT01508247). Written informed consent was obtained from parents or guardians of each subject. Independent Ethics Committee approvals were obtained from the Ethics Committee of the Jiangsu Provincial Center for Disease Prevention and Control. 165 serum samples were collected from healthy adults (18–65 years old) in a Hepatitis A virus vaccination cohort, which were approved by China Food and Drug Administration (Clinical research document No. 2012L00668) and Ethics Committee of the Jiangsu Provincial Center for Disease Prevention and Control. Written informed consent of each participant was obtained.

2.3. CVB3 single round infection system

2.3.1. CVB3 sub-genomic replicon

CVB3 replicon was constructed by replacing the capsid coding region with a firefly luciferase reporter gene on CVB3-Nancy, an infectious viral cDNA clone kindly provided by Dr. Jeffrey M. Bergelson (Pan et al., 2011). A 2A protease cleavage site (AITTL) was inserted between luciferase gene and 2A. CVB3 replicon was linearized with MluI and 1 μg phenol–chloroform extracted DNA was then used as template for CVB3 replicon RNA synthesis with RiboMAX large scale RNA production kit (Promega) following the manufacturer's instructions. Transcribed RNA was purified by isopropanol precipitation and used for transfection or frozen at −80 °C until use.

2.3.2. CVB3 capsid expressers

The CVB3 (Nancy) capsid expresser was constructed on pcDNA6.0A and expressed the capsid genes *in trans*. EGFP gene was inserted upstream of the CVB3 (Nancy) capsid gene, and was separated by a 2A protease self cleavage site (AITTL). The EGFP reporter is used for monitoring transfection efficiency and expression level of the structural genes.

2.4. Preparation of CVB3 pseudovirus-CVB3 (Nancy)-luc

CVB3 (Nancy)-luc was produced by sequential transfection of CVB3 capsid expresser plasmid and CVB3 replicon RNA into HEK-293T cells. Briefly, CVB3 (Nancy) capsid expresser was reverse transfected into HEK-293T cells at 80% confluence with jetPRIME® (Polyplus); 24 h post capsid transfection, replicon RNA was then transfected with Lipofectamine 2000™ (Life technologies). CVB3 (Nancy)- luc pseudovirus was harvested at 24 h post RNA transfection with 2 rounds of freeze–thaw cycle. Pseudovirus was stable at −80 °C for at least six months without significant infectivity decrease.

2.5. Quantification of CVB3 (Nancy)-luc pseudovirus with qPCR

Viral RNA was extracted from 10 μL pseudovirus sample with QIAamp Viral RNA Mini Kit (Qiagen) and cDNA was synthesized with PrimeScript RT Reagent Kit (Takara). Viral titer was quantified by measuring the genome copy equivalents with qPCR using SYBR Premix Ex Taq II (Perfect Real Time) (Takara). The primers targeted to firefly luciferase reporter gene were used for qPCR quantification (qLuc-F: 5′-caaatacgatttatctaatttacacga-3′; qLuc-R: 5′-ccggtatccagatccacaac-3′).

2.6. Characterization of CVB3 pseudovirus by ultracentrifugation

Equal amount of CVB3 (Nancy) wild type virus and pseudovirus (250 μL) were mixed and loaded on a 15–30% discontinuous sucrose gradient, followed by ultracentrifugation at 40,000 rpm for 70 min at 10 °C with Beckman MLS-50 rotor. 10 μL of each fraction samples (total 10 fractions) were subjected to RT-qPCR. The copy number of viral genomic RNA in each fraction was subsequently determined by RT-qPCR. CVB3 (Nancy) virus was quantified with primers located in VP1 (qVP1-F: 5′-cagtgtgttttggaccgagg-3′; qVP1-R: 5′-agcgtgcccatgttgtttag-3′); specific primers for firefly luciferase reporter gene (qLuc-F and qLuc-R) were used for quantifying CVB3 (Nancy)-luc pseudovirus.

2.7. Titration of CVB3 (Nancy)-luc pseudovirus

The pseudovirus titer was determined by measuring the 50% cell culture infective dose (CCID50) using a microtitration assay. 200 μL two fold series diluted CVB3 (Nancy)- luc was added to 96-well plate in duplicates and 100 μL Hela cell suspension (2×10^4 per well) was added later. After incubation at 37 °C for 12–15 h, the cells were lyzed in passive lysis buffer to measure luciferase activity following the manufacturer's instructions (Promega). Luciferase activity measured in relative light units (RLUs) was determined and

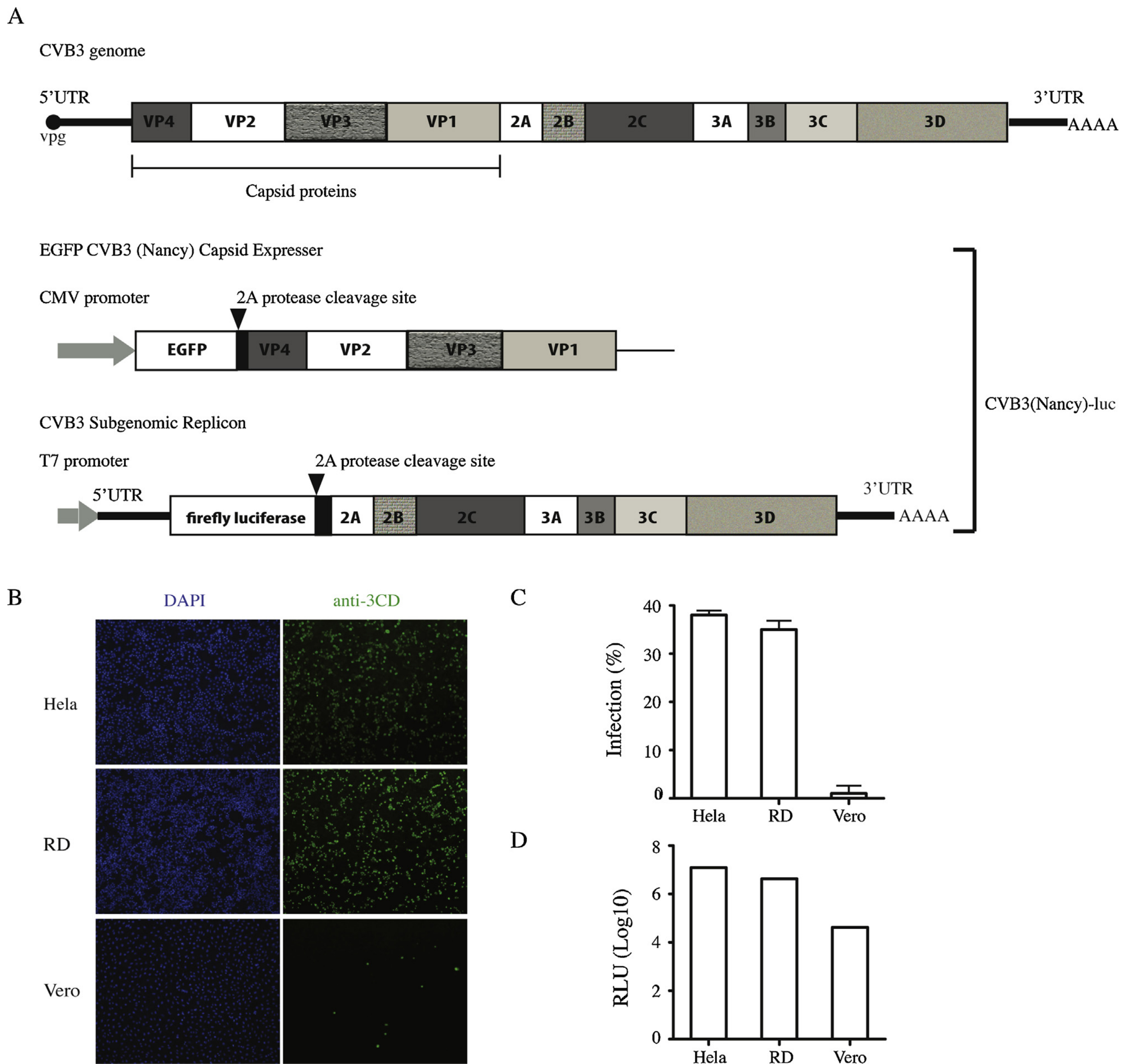

Fig. 1. Single round CVB3 infection system. (A) Schematic map of CVB3 viral RNA genome, CVB3 replicon and capsid expresser for single round CVB3 infection system. The CVB3 capsid expresser was used to express all the structural capsid genes *in trans*, an EGFP gene was inserted upstream of the CVB3 (Nancy) capsid gene and all other CVB3 viral genome were deleted, the EGFP was separated by EV71 2A self cleavage site (-AITTL-) from the structural genes; CVB3 replicon was produced by replacing the capsid coding region with a firefly luciferase reporter gene in the full length CVB3 genome and a T7 promoter was placed at the 5′ end for transcription *in vitro*. (B, C) Relative infectivity of CVB3 (Nancy) wild type virus on Hela, RD and Vero cells. (B) Immunostaining of CVB3 Nancy strain wild type virus infected cells. Cells with 80% confluence on plates were infected with Nancy virus at 100 $TCID_{50}$ and stained with mouse anti-3CD serum at 24 h post infection (C) The infection ratio was quantified with Columbus™ (PerkinElmer). (D) Relative infectivity of CVB3 (Nancy)-luc virus on Hela, RD, and Vero cells. Cells were infected with 1 μL CVB3(Nancy)-luc virus (~2×10^6 copies), luciferase activity was measured at 24 h post infection.

cells which had RLUs 10 times above the background were considered positive of pseudovirus infection. The background control was cells without pseudovirus infection.

2.8. Immunostaining of CVB3 infection

2.8.1. Purification of CVB3 (Nancy) 3CD recombinant protein

cDNA coding full length Nancy 3CD pro precursor was cloned to pET28a. Transformed BL21 (DE3) cells were induced with 100 μM IPTG at 16 °C for 20 h in LB (Kan^+). Recombinant protein was purified with Ni-NTA agarose beads (Qiagen). The purified 3CD protein was then concentrated, aliquoted, and stored at −80 °C.

2.8.2. Mice immunization

3CD proteins were emulsified with equal volume of complete Freunds adjuvant (for first immunization) or incomplete Freunds adjuvant (for booster) and 6–8 weeks-old BALB/c mice were immunized at 4 weekly intervals (20 μg/mouse). Serum was collected at one week after the third booster.

2.8.3. Immunostaining of CVB3 (Nancy) wild type virus infection

Cells were fixed and permeabilized with ice-cold methanol for 10 min at Room temperature at 12 h post CVB3 (Nancy) infection, then washed with PBS and incubated with mouse anti-3CD polyclonal antibodies diluted in 2% BSA/PBS for 1 h at 4 °C. After 3 times of washes with PBS, the cells were incubated with Donkey anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 488 con-

jugate (1:1000 diluted in 2% BSA/PBS) for 0.5 h at 4 °C. After 3 times of washes with PBS, anti-fading reagent was added before observation under fluorescent microscope (Nikon). Fluorescent images were analyzed with Columbus™ (PerkinElmer).

2.9. Neutralizing assay based on pseudovirus system expressing firefly luciferase

Sera were heat-treated at 56 °C for 30 min to inactivate complements. Two-fold serial dilutions of sera from 1:10 were made, 50 μL diluted serum was taken out and incubated with equal volume of diluted pseudovirus at 37 °C for 1 h in 96-well plates. Then 100 μL Hela cell suspension (2×10^4 per well) was added into the wells and incubated at CO_2 incubator. After incubation, medium supernatant was discarded and cells were lyzed in passive lysis buffer (Promega) with two rounds of freeze–thaw cycles, and stored at −80 °C before measurement. Luciferase activity measured in relative light units (RLUs) was determined according to the luciferase assay system user's manual (Berthold). Inhibition ratio was calculated as: $[1-(RLU_{\text{virus incubated with serum}} - RLU_{\text{background}})/(RLU_{\text{virus control}} - RLU_{\text{background}})] \times 100$. And non-linear regression curve was plotted with logarithm-10 transformed dilution concentration against inhibition ratio by Graphpad. The titer of neutralizing antibodies was determined as the reciprocal of the dilution at which 50% of the complete pseudovirus neutralization (pNT50).

2.10. Microtiter plate neutralization assay based on CPE

In brief, two-fold serial diluted sera were mixed with an equal volume (50 μL) of virus working solution containing 100 TCID50/well (50% of tissue culture infective dose) of CVB3 strain 112 (DH16G/JS/2012, Genbank accession NO. KP036480) at 37 °C for 1 h in 96-well microtiter plates. 100 μL Vero cells (1×10^4 per well) were added and incubated at 37 °C for 7–9 days. Sera were tested in duplicates. The neutralization titer was determined as the reciprocal of the highest dilution at which over 50% of wells showed complete inhibition of CPE.

2.11 Statistical analysis

Results were obtained from at least duplicates and reported as the mean ± standard deviation (SD). All statistical analyses were performed with the GraphPad Prism software package.

3. Results

3.1. Generation of CVB3 (Nancy)-luc pseudovirus for single round infection

We first established a single-round CVB3 reporter virus system which could produce CVB3 pseudovirus with high titer and robust infectivity. Similar strategies had been applied based on our experiences in previously established EV71 single round infection system (Chen et al., 2012). CVB3 sub-genomic replicon plasmid (CVB3 replicon) contains untranslated regions (UTRs), firefly luciferase (luc) reporter gene and all nonstructural protein regions, with a T7 promoter sequence at the upstream of 5′ end used for *in vitro* transcription. The capsid expressing plasmid, which is designated as EGFP CVB3 (Nancy) Capsid Expresser, contains all the structural genes (VP1-4) and an EGFP reporter gene that are under control of a CMV promoter (Fig. 1A). A 2A protease cleavage sequence (AITTL) which was from EV71 genome were introduced both in CVB3 replicon (between luciferase gene and nonstructural genes) and EGFP CVB3 (Nancy) Capsid Expresser (between EGFP gene and capsid). Efficient protease cleavage upon viral polyprotein translation in these two sites was required for viral proteins maturation and pseudovirus encapsidation. The single round pseudovirus was produced by sequential transfection of the capsid expressing plasmid and CVB3 sub-genomic RNA transcribed from CVB3 replicon *in vitro* (see Section 2). The produced virus is designated as CVB3 (Nancy)-luc. These pseudoviruses can only infect cells once as they lack structural genes and their capsid was provided in producing cell *in trans*. Successful production of high titer CVB3 pseudovirus showed that inserted 2A sequence from EV71 could be efficiently recognized by CVB3 viral proteases.

We next compared CVB3 (Nancy)-luc with wildtype virus in respects of tropism, virion structure and antigenicity. The sensitivity to different cell lines was the same as wild type CVB3 Nancy strain (Fig. 1B–D). In an ultracentrifugation analysis, CVB3 (Nancy)-Luc pseudoviruses migrated the same as the wild type virus in a discontinuous 15–30% sucrose (Fig. 2A), and this showed that pseudovirus particles had the same sediment coefficient as wild type virions. And CVB3 (Nancy)-Luc infection could be neutralized with mouse anti-CBV3 serum in a dose-dependent manner (Fig. S1). We next verified that antiserum from mice immunized with inactivated Nancy viruses could specifically neutralize the infection of CVB3 (Nancy)-luc, while WHO standard antisera against Polio virus, goat anti-G10/CA16 serum, mouse anti-FY523/EV71 serum, mouse anti-Coxsackievirus B5 (CVB5) serum, mouse anti-Hepatitis A virus (HAV) could not neutralize either CVB3 (Nancy)-luc (Fig. 2B) nor CVB3 (Nancy) wild type virus (data not shown) infection *in vitro*. Taken together, these results proved that the singe-round CVB3 (Nancy)-luc pseudovirus system could be used as a surrogate for wild type virus. This pseudovirus not only provides the excellent tool for studying CVB3 entry process in basic research by avoiding any ambiguity that might be caused by post entry events, but also has some other clinical applications. Previous neutralization result showed good prospect in utilizing CVB3 (Nancy)-luc to detect neutralizing antibodies in clinical sera, which is a significant indicator for vaccine efficacy. We next explored the feasibility to develop an *in vitro* neutralization assay based on this CVB3 pseudovirus expressing firefly luciferase.

3.2. Optimization of pseudovirus luciferase assay

Time course experiment was done to find optimal incubation time post CVB3 (Nancy)-luc infection and showed that RLUs was rather stable between 9 h and 18 h post infection, while significantly decreased after 24 h post infection (Fig. 3A). Linearity between relative light units (RLUs) and CVB3 (Nancy)-luc input (showed as genome equivalent) was analyzed and the result was shown to be quantitative, with linear correspondence between luciferase activity and the amount of input virus over a broad range (Fig. 3B). CCID50 of CVB3 (Nancy)-luc had also been determined, a dose of 200CCID50 pseudoviruses per well which had been in the linear correspondence were used for the following experiments. Reproducibility is an important criterion to be considered. For the lack of CVB3 national antibody standard, we used a mouse serum collected from mice immunized with formalin inactivated CVB3 virus 112 strain and quantified its neutralizing activity against CVB3 (Nancy)-luc pseudovirus in duplicate on the same plate in six independent tests. Pearson correlation analysis of neutralizing results from six independent tests showed high degree of reproducibility ($r^2 > 0.98$), while the pNT50 of this mouse serum was 108.9 ± 19.35 (Fig. S1).

3.3. Comparison of pseudovirus luciferase assay with traditional CPE based assay

Previously we have measured neutralizing antibodies titers of 720 serum samples collected from pre-school participants (14

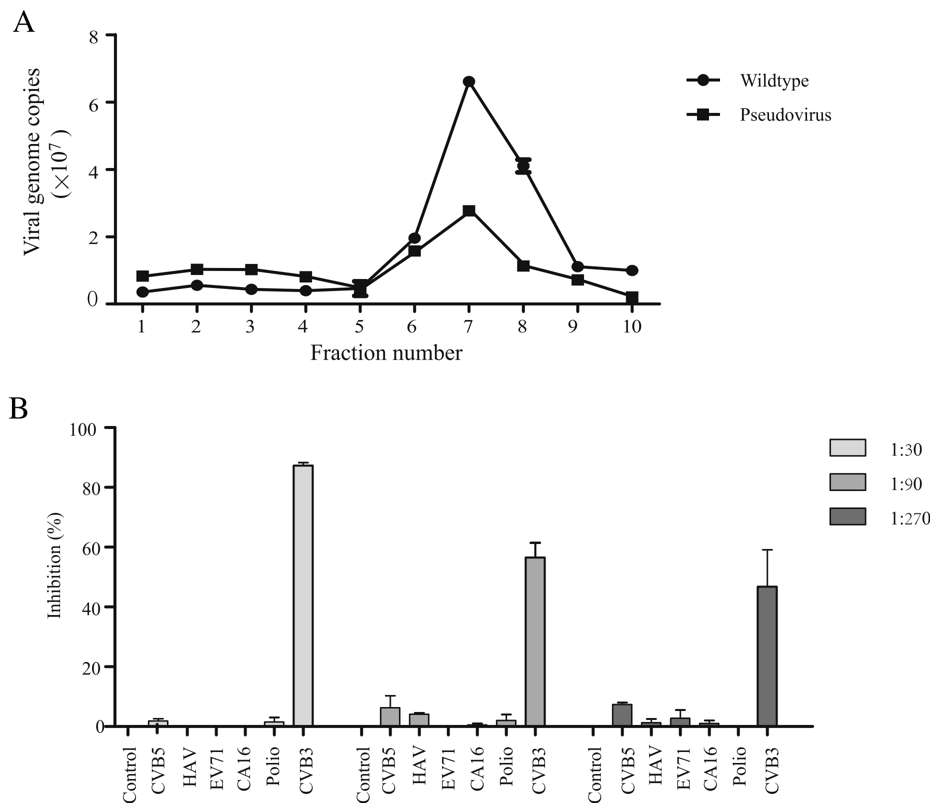

Fig. 2. Characterization of CVB3 (Nancy)-luc pseudovirus. (A) Ultracentrifugation analysis of CVB3 (Nancy)-luc pseudovirus. Both CVB3 (Nancy) wild type virus and pseudovirus were mixed and applied on a sucrose gradient. After ultracentrifugation at 40,000 rpm for 70 min at 10 °C with Beckman MLS-50 rotor, the copy number of viral genomic RNA in each fraction was subsequently determined by quantitative RT-PCR. Wild type virus was quantified with primers located in VP1, while CVB3 (Nancy)-luc pseudovirus with primer located in firefly luciferase reporter gene. (B) Specific neutralization of CVB3 (Nancy)-luc with antisera. Antisera including mouse anti-coxsackievirus B5 (CVB5) serum, mouse anti-hepatitis A virus (HAV), mouse anti-FY523/EV71 serum, goat anti-G10/CA16 serum, WHO standard antisera against Polio virus, mouse anti-coxsackievirus B3 (CVB3) serum were serially diluted and incubated with CVB3 (Nancy)-luc at 37 °C for 1 h prior to infection of Hela cells in 96-well plate in a total volume of 100 μL in quadruplicates. Luciferase activity was measured at 16 h post infection.

months to two years old) in a phase III clinical trial on inactivated EV71 vaccine using traditional CPE assay. Then we applied our newly established pseudovirus assay to re-analyze the titers of these samples, and compared the results with the CPE data. We used capsid protein of CVB3 prototype Nancy strain to encapsidate pseudovirus. Although sequence alignment of capsid proteins revealed several mutations in 112 strain compared with Nancy strain (Fig. S2), the neutralizing data using both assays were highly consistent, no obvious divergence in antigenicity of 112 and Nancy strains had been observed.

As there is no antibody standard reference with known concentration (Units) for CVB3, we could not determine antibody concentration with units; instead we used the reciprocal of the dilution at which 50% of the complete pseudovirus neutralization (pNT50). As for the traditional CPE assay, neutralization titer was determined as the reciprocal of the highest dilution at which over 50% of wells showed complete inhibition of CPE (CCID50), samples with titer ≥8 were considered positive, otherwise were negative. Two sets of data were then underwent statistical analysis. 6.39% (46/720) of the samples were positive in pseudovirus assay compared with 6.8% (49/720) were positive in CPE assay. Spearman correlation analysis showed that there was good correlation between the results from these two assays (spearman $r = 0.804$, $P < 0.0001$) (Fig. 3C). Bland–Altman method comparison analysis further showed that these two methods were highly consistent. The average of logarithmic difference for quantitative results of the two methods was 0.7157, and the standard deviation was 0.3193 (Fig. 3D).

3.4. Seroprevalence of CVB3 in pre-school children and adults

We next used this pseudovirus assay to measure CVB3 neutralizing antibodies titers in serum samples collected from health adults (18–65 years old). 60% of serum samples (99/165) were CVB3 seropositive. 146 infant participants, whose serum samples were collected within the same period as the adult participants, were picked out for seroprevalence analysis. We found the pre-school group has significantly lower positive ratio (11.94%, 19/159) than adults. The geometric mean of neutralizing antibodies titer in pre-school children was 59.29 (95% CI: 42.96–174.7), while in adults it was 65.91 (95% CI: 80.34–150.4). Although unpaired t test analysis showed that the difference in titer of these two groups was not statistically significant, the geometric mean titers were slightly higher in adults group (Fig. 4). More detailed seroprevalence pattern would be discovered if the population can be further stratified into more specific groups according to their ages or gender.

4. Discussion

Trans-encapsidation methods have been used to produce pseudoviruses for picornaviruses. Poliovirus replicon was able to pseudotype some picornaviruses when their capsid proteins were provided *in trans*, though with variable efficiency; Among these viruses, CVB3 was the most efficient in trans-encapsidation of the poliovirus replicon (Jia et al., 1998; Porter et al., 1998). In our previous work, we found CA16 could be pseudotyped with EV71 replicon with moderate efficiency; however CVB3 failed to be pseudotyped (Fig. S3). So we established a CVB3 single round infection system

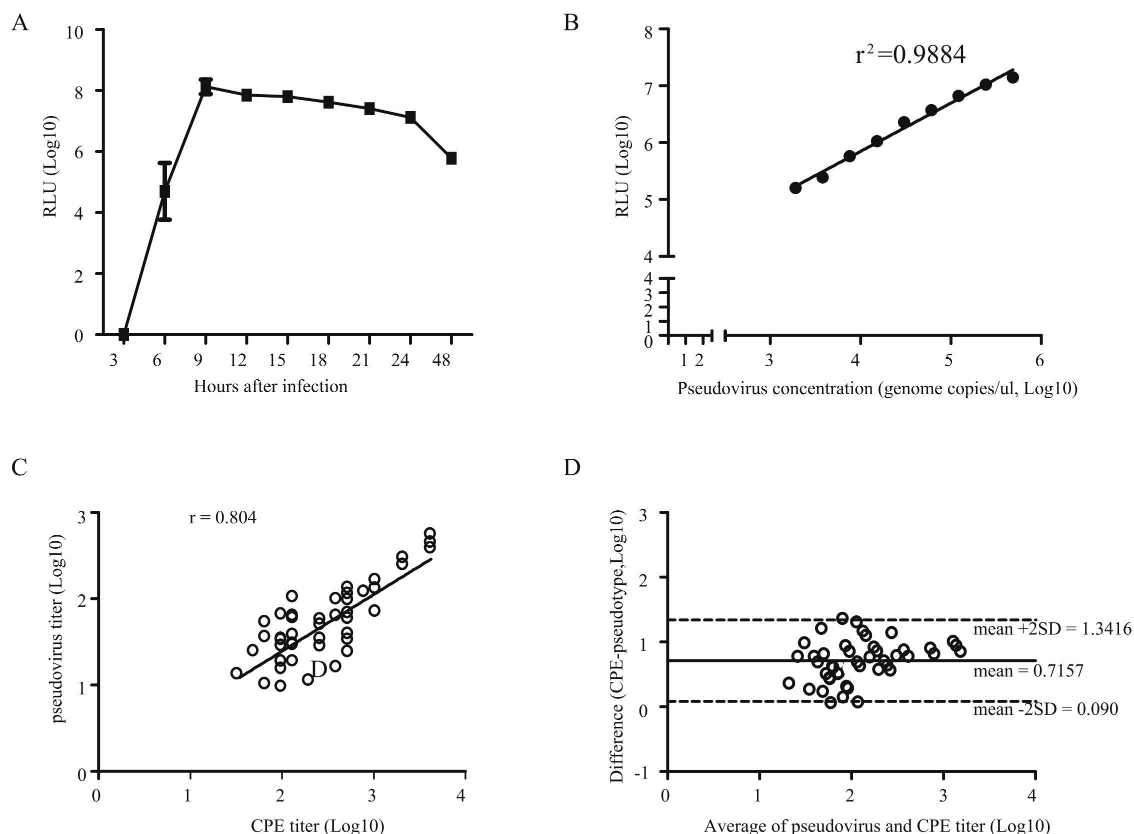

Fig. 3. Optimization CVB3 (Nancy)-luc based pseudovirus neutralization assay. (A) Time course of CVB3 (Nancy)-luc infection. Hela (2×10^4 cells per well) cells were infected with 1 μL CVB3 (Nancy)-luc virus ($\sim 2 \times 10^6$ copies) on 96 well plates, luciferase activities were measured at 3–24 h with 3 h interval as well as 48 h post infection. Each data point represents the average of eight replicates. (B) Linearity of CVB3 (Nancy)-luc infection system. Hela cells (2×10^4 cells per well) were incubated with serially diluted CVB3 (Nancy)-luc virus (from 2×10^3 to 5×10^5 per μL). Luciferase activities were measured at 12 h post infection. Each data point represents the average of eight replicates. Linear regression analysis was performed using Graph Pad Prism. Points that beyond the linear range were excluded. (C) Spearman correlation analysis of CVB3 neutralizing antibodies titers in human serum samples measured both by CPE assay and pseudovirus assay. 46 pre-school children serum samples were considered positive in both assays. As titers of two samples were beyond the measurable range of CPE assay (titers >1024), titers of the other 44 samples were used in the following analysis. Data analysis was performed using Graph Pad Prism. The solid line represents the linear regression curve. ($r = 0.804$, $p < 0.0001$). Each hollow dot represents a seropositive pre-school child. (D) Bland–Altman method comparison analysis of CVB3 neutralizing antibodies titers in human serum samples measured both by CPE assay and pseudovirus assay. The solid line represented the mean value, while dashed lines represented the 95% confidence limits. Each hollow dot represents a seropositive pre-school child.

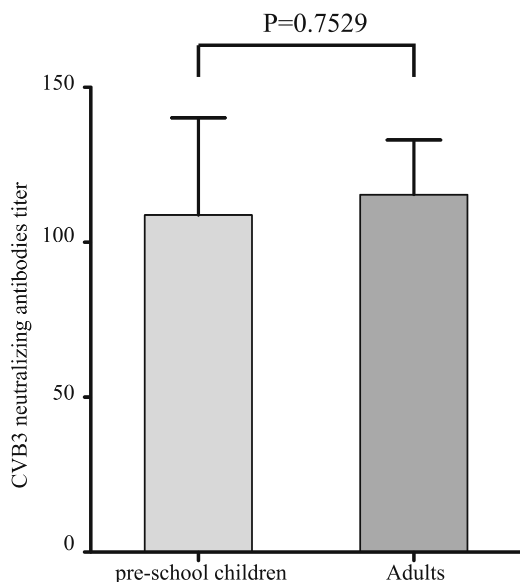

Fig. 4. Comparison of CVB3 neutralizing antibodies titers in pre-school children and adults. CVB3 neutralizing antibodies titers of 19 pre-school children and 99 adults were included in unpaired t test analysis ($P = 0.7529$). The geometric mean of neutralizing antibodies titer in pre-school children was 59.29 (95% CI: 42.96–174.7), while in adults it was 65.91 (95% CI: 80.34–150.4). Data analysis was performed using Graph Pad Prism.

with high titers using CVB3 replicon, and found that neither EV71 nor CA16 could be pseudotyped with CVB3 replicon (Fig. S3). Different compatibility among picornaviruses suggested that there might be encapsidation signal either on replicon RNA or *in trans* provided capsid proteins or both, and common determinants might be found if viruses were classified into categories according to their pseudotyping compatibility.

Our work also developed a quantitative pseudovirus luciferase assay for detecting neutralizing antibodies in clinical serum samples. This assay was proven to be safe, fast, sensitive and showed good correlation with traditional CPE based assay. This assay would greatly facilitate CVB3 vaccine development and evaluation as well as seroprevalence survey.

Besides the above application, this assay could also be used in screening for anti CVB3 human monoclonal antibodies and entry inhibitors which would be potent antiviral drugs against CVB3 infection. Although only capsid of Nancy strain was tested in this work, other subtypes of CVB3 are ready to be pseudotyped with their capsid proteins. Cross neutralizing activity of antibodies could be easy to be analyzed and broad neutralizing antibodies with therapeutic value could thus be identified, as well as potential differences in antigenicity could be analyzed which would contribute to vaccine candidate selection.

Increasing CVB3 infection cases have been reported in HFMD surveillance and changes in HFMD pathogen spectrum have been

noticed. After successful development of effective inactivated whole-virus EV71 vaccines, EV71 infection cases would drop significantly in the future, while CVB3 might be a prominent enterovirus replacing EV71. Serological investigation of neutralizing antibodies in the population would allow us to assess the protection level against viral infections. In this work, we also did a small scale serological survey of CVB3 in health adults, and compared CVB3 seroprevalence in pre-school children and adults. The existence of neutralizing antibodies reflects previous infections. The higher positive rate of CVB3 neutralizing antibodies in adults indicates that there have been CVB3 infections during childhood. Despite the different geographic sampling, the serological pattern is the same as the investigation in Yantai city of China (Tao et al., 2013). Low positive rate of CVB3 neutralizing antibodies in infants indicated these infants were naïve to CVB3 and with higher risk in potential CVB3 outbreaks. Not only CVB3, but also many enteroviruses including EV71, CA16, CA6 have been reported with higher positive rate and titers of neutralizing antibodies in adults, while low positive rate in infants or pre-school children which makes them susceptible to enteroviruses infection (Ji et al., 2012; Ang et al., 2015).

We found that there was no cross neutralization between CVB3 and selected enteroviruses (EV71, CA16, CBV5, PV, HEV), using sera collected from mice immunized with inactivated viruses (Fig. 2B); moreover, we previously measured neutralizing antibodies titers against EV71 in the 720 serum samples collected from pre-school children, and found no statistically significant correlation of neutralizing antibodies titers against EV71 and CVB3 (data not shown), and this observation indicated that there were also no cross neutralization between CVB3 and EV71 in human. Taken together, we propose that neutralizing antibodies against EV71 and CVB3 can be measured simultaneously. We next would substitute firefly luciferase reporter in CVB3 replicon with renilla luciferase reporter, and use both our previously developed EV71 pseudovirus expressing firefly luciferase and CVB3 pseudovirus expressing renilla luciferase to measure neutralizing antibodies against each virus in the same well (Duel pseudovirus expressing luciferase reporter system). This would further minimize the cost and required clinical serum volume in practice. Many enteroviruses have been found to be associated with HFMD in previous HFMD surveillance; multivalent vaccine may be needed for more effective HFMD control (Liu et al., 2014). Assays with the capacity to evaluate neutralizing antibodies to multiple pathogens simultaneously would be preferred in the future.

In summary, we established a single round infection system of CVB3 and developed an *in vitro* assay for detecting neutralizing antibodies in clinical serum samples, and it was a superior surrogate of the assays using wild type viruses including traditional CPE assay and enzyme-linked immunosorbent spot assay. We also analyzed the differences in CVB3 seroprevalence in two representative population groups and found that most pre-school children were naive to CVB3. Although more serum samples from diverse geographic areas and different ages are needed to get a more detailed view of CVB3 seroprevalence, our work provided evidences that CVB3 might impose potential public health threats and further efforts are needed for CVB3 infection control.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2016.02.010.

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